

# **Biofuels Program Semiannual Report**

## **First Half FY 2000**

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## **PRETREATMENT TECHNOLOGY**

### **Summary of Technical Achievements or Results**

Work within the Pretreatment Technology Team during this reporting period was focused in four interrelated areas.

- 1) Shakedown of the engineering-scale countercurrent pretreatment reactor system was completed and initial operation of the full system under targeted reaction conditions has begun.
- 2) Work continues on evaluating the process operability issues associated with the proposed reaction conditions of the countercurrent pretreatment process, with an emphasis on gathering information needed to quantify the significance of the operability issues upon process economic projections and the ultimate feasibility of this approach.
- 3) We began initial limited investigation which should lead to a more fundamental understanding of hydrolysis chemistry mechanisms. This, in turn, may help us identify a re-configured process where less severe conditions than the present countercurrent process may be possible, directly impacting some of the process operability concerns.
- 4) We conducted initial bench-scale testing and identified a process scenario and process equipment for an improved prehydrolysis process, based on the concept of hot separation and washing of the pretreated solids.

### **General Technical/Scientific Progress**

#### **Shakedown and Initial Operations of Engineering-Scale Countercurrent Pretreatment Reactor System**

The mechanical installation of the engineering-scale countercurrent pretreatment reactor system was completed in September 1999, which was immediately followed by shakedown of the system. The shakedown activities involved testing the operation of individual systems, first under ambient conditions and eventually under actual operating conditions that the particular individual system will need to operate at during full system operations. Several modifications or equipment changes were made in the system as a result of the lessons learned during the shakedown of the individual systems, but all such changes were relatively minor and were accomplished in a short time frame.

We completed the shakedown of the individual systems was in January 2000, which led directly to the initial operations of the entire system under targeted operating conditions. Four full system runs were conducted between January and March, 2000. After each run, we evaluated how the reactor system operated during the run, then we conducted a series of equipment and/or procedural modifications.

By the end of this reporting period, we have learned how to achieve countercurrent movement of solids and liquids in the vertical stage reactor under reaction conditions and how to start-up and operate the system to collect process data and process samples needed for determining process yields and mass balance closure. We are now focussing on operating the system in a consistent and reproducible manner to determine whether the key system performance parameters (process yields, liquid/solid ratio, and mass balance closure) are achievable.

In addition, the draft Standard Operating Procedure (SOP) for the engineering-scale countercurrent pretreatment reactor system was updated with a complete set of detailed operating procedures. This final SOP is now being reviewed by the National Renewable Energy Laboratory's (NREL's) Environment Safety and Health department.

## **Process Operability Issues Associated with Proposed Countercurrent Pretreatment Reaction Conditions**

The countercurrent pretreatment reaction configuration and conditions that have been proposed include a steam co-current first stage operated at 183°C /150 (psig) followed by a countercurrent second stage using 0.07% sulfuric acid operated at 225 °C /370 psig. The high temperatures and pressures proposed in the second stage have led to some concerns regarding the commercial practicality and operability of the process as a result of these conditions. An on-going subcontract with the Harris Group (Seattle, WA) that is investigating pretreatment reactor design, equipment, and cost is also investigating the specific issues associated with the proposed countercurrent pretreatment reaction conditions. The goal is to first determine whether equipment designs exist that can operate under the proposed conditions and to determine the impact of the higher temperature and pressure on reactor component cost, lifetime, and maintenance requirements. This follows up the workshop held at NREL in April 1999 involving industrial experts who have experience with feeding biomass-type feedstocks into and through pressurized reactors at a commercial-scale. The workshop focused on the ability to feed biomass feedstocks into the high-pressure reaction conditions in the countercurrent stage at the proposed reaction conditions. Both the workshop panel and the preliminary findings from the Harris Group determined that several feeder systems are available that may be applicable to biomass hydrolysis processes, although all appear to suffer some limitations and none have been actually operated in a commercial setting under such conditions. The Harris Group is now extending the evaluations to provide information on the potential cost impact of operating under these conditions and to determine what type of process demonstration might be required for an engineering firm to be able to issue process performance guarantees.

## **Fundamental Understanding of Cellulose and Hemicellulose Hydrolysis Mechanisms**

In an attempt to mechanistically explain the high yields achieved in the bench scale-shrinking bed simulated countercurrent apparatus (>90% yield of glucose from cellulose), we are developing a more fundamental understanding of the hydrolysis chemistry mechanisms involved. This has led us to a mechanism that may allow us to reconfigure the proposed countercurrent process with less severe, i.e. lower temperature/pressure conditions while still being able to achieve high yields of glucose from cellulose. A hypothesis based upon a heterogeneous reaction mechanism and the requirement to disrupt a water-molecule boundary layer has been developed to explain the superior cellulose hydrolysis and glucose yields achieved in the present configuration of the countercurrent pretreatment process. If this hypothesis is correct, there may be other chemical methods to disrupt this boundary layer besides the scouring effect of the flowing countercurrent, shrinking-bed process. This in turn, may allow us to operate the process at lower temperatures and/or pressures, which would directly impact some of the equipment-related operability concerns. A subcontract has been competitively awarded to J. Brady (Cornell University) to use molecular modeling tools to determine if the proposed water-molecule boundary layer exists under reaction conditions and if its disruption can lead to a higher-yielding cellulose hydrolysis process under lower temperature and/or pressure conditions. Initial information necessary to prove or disprove the boundary layer hypothesis is expected to be available by September, 2000.

## **Improved Prehydrolysis/Hot Separation and Washing Process Development**

We have developed an approach to determine the process and economic potential of improved prehydrolysis, based on some of the reactor design virtues of the countercurrent pretreatment approach. This concept involves a standard single-stage co-current dilute acid prehydrolysis followed by separation of the pretreated slurry into liquid and solid streams and washing of the pretreated solids at an elevated temperature (above 135°C). The separation and washing of the pretreated solids at elevated temperatures is believed to prevent the re-precipitation of lignin that is solubilized in the prehydrolysis reaction back upon the pretreated solids, resulting in pretreated solids that are more

easily and completely digested by cellulase enzymes in the subsequent SSCF unit operation.

We have found a commercially available separator/washer capable of operating under the specified conditions through the on-going process engineering subcontract with the Harris Group. Initial evaluation of a laboratory test unit for this equipment has been conducted. This unit provides test data that can be utilized in scale-up calculations to the pressurizeable continuous belt filter manufactured by Pneumapress Inc. (Richmond, CA). This unit was plumbed directly to a modified 1 liter Parr reactor that allowed for direct transfer of the hot pretreated slurry to the filtration test unit. A single-stage batch prehydrolysis using 0.07% (w/v) sulfuric acid at 204 °C for 10 minutes was followed by transfer to the filtration and washing test unit under pressure, with separation of the pretreated slurry into liquid and solid streams and washing of the solids at about 150 °C.

The test unit from Pneumapress is virtually identical in design to our 2-inch-by-12-inch percolation reactors. Based on the successful initial tests, we have modified one of our percolation reactors to serve as a hot filtration and washing test unit and are conducting a designed experiment to determine washing temperature and wash volume requirements needed to produce the highly digestible pretreated solids. Ultimately, this data will be incorporated into process engineering models to determine whether qualification of opportunity performance targets can be achieved.

## **Scientific Publications, Presentations, and Other Activities**

### **Patents Issued**

Torget, R., Padukone, N., Hatzis, N. and C. Wyman. U.S. Patent No. 6,022,419. (8 February 2000). Hydrolysis and Fractionation of Lignocellulosic Biomass.

### **General Presentations/Travel**

R. Elander and R. Torget gave a presentation to the DOE Office of Fuels Development Staff at NREL on December 14, 1999.

R. Elander and T. Torget met with M. Antal of the University of Hawaii at NREL on January 10, 2000.

R. Elander, R. Torget, and N. Nagle gave a presentation to and reviewed the status of the countercurrent pretreatment reactor system with A. Mokvist of Sunds Defibrator Inc. (now Valmet Corp.) on March 21, 2000.

R. Torget became a charter member and attended meetings of the newly-formed Biomass Refining Consortium in Applied Fundamentals and Innovation (CAFI) in November 1999 and February 2000. Other membership of the consortium includes B. Dale (Michigan State), M. Holtzapfel (Texas A&M), M. Ladisch (Purdue), Y.Y. Lee (Auburn), and C. Wyman (Dartmouth).

### **Scientific Meetings: Papers and Posters Presented or Recently Accepted for Presentation**

The following presentations were accepted for the 22nd Symposium on Biotechnology for Fuels and Chemicals in Gatlinburg, TN, May 7-11, 2000:

A poster presentation, "Kinetics of Dilute-Acid Catalysis and Autohydrolysis of Hardwood Xylan" by R. Torget, R. Elander, Y.Y. Lee (Auburn University) and J.S. Kim (Auburn University).

A poster presentation, "Investigation of Hot Separation and Washing Techniques to Produce Highly

Digestible Pretreated Solids Following Dilute Acid Prehydrolysis of Hardwood" by R. Elander and R. Torget.

#### **Scientific Journals: Papers Accepted for Publication**

Lee, Y.Y., Wu, Z. and R. Torget. (2000). "Modeling of Countercurrent Shrinking-Bed Reactor in Dilute-Acid Total Hydrolysis of Lignocellulosic Biomass" *Bioresource Technology* (71), pp.29-39.

#### **Scientific Journals: Papers Submitted for Publication**

A paper entitled "Fundamental Aspects of Dilute-Acid Hydrolysis/Fractionation Kinetics of Hardwood Carbohydrates - Part I: Cellulose Hydrolysis" by R. Torget, J.S. Kim (Auburn University) and Y.Y. Lee (Auburn University) was submitted to *Industrial & Engineering Chemistry Research*.

#### **Project Operation**

A subcontract entitled "Modeling of Solvent Structuring Near Microcrystalline Substrate and Its Effects on Acid Hydrolysis" was awarded to Cornell University (Dr. John Brady, Principal Investigator) and was initiated in March 2000. The 12-month subcontract is funded with \$25,000 in FY 2000 funds, with an approximate additional \$25,000 planned for FY 2001.

The subcontract entitled "Lignin Recovery and Upgrading, Kinetic Modeling" being performed by Auburn University (Dr. Y.Y. Lee, Principal Investigator) was granted an extension through April 30, 2000, at an added cost of \$4,500.



## IMPROVED CELLULASE ENZYMES

### Summary of Technical Achievements or Results

The robotics facility at NREL has now been equipped with two decks from Cyberlab, Inc. Using the C-300 deck, we will conduct first-tier screening of cellulase activities using chromophoric or fluorophoric substrates. The second deck (C-400) was designed by NREL specifically for the automated filter paper assay, but will also serve to conduct second-tier screening of modified cellulases. This deck performs quantitative sample dilution, automated plate sealing, incubation with filter paper coupons at low temperature, dinitrosalicylic acid reagent addition, color development at a higher temperature, automated OD detection, and plate inventory.

We are progressing in developing an assay to probe the strength of binding of native EI and rEI surface charge mutants to. We are measuring the fraction of EI enzyme bound to substrate (versus free enzyme) as a function of time and temperature. Changing the reaction vessel corrected initial mixing problems. Addition of 100 mM NaCl and 150 ( g/ml) BSA to the reaction buffer improved the reproducibility significantly, but BSA was found to compete with the enzyme for binding sites. Buffers now contain only the NaCl. Standard deviation is now about 5% on a consistent basis. This includes data from both the pNPC activity assay and the BCA reducing sugars assay. The effect of protein surface charge on the strength of binding to cellulose is currently unknown.

We recently analyzed several commercial and NREL Enzymatic Process Development enzyme preparations for filter paper activity and cellulase protein content-- these include preparations from Genencor Intl., Dyatic, Rhodia, and Iogen. We prepared these samples for analysis by first desalting 5 (mL) using FPLC into 20 mM acetate, pH 5.0 buffer and then applying the standard filter paper units (FPU) assay and the Pierce Micro BCA protein assay. To date, we have found no cellulase preparation with greater than 600 FPU/g cellulase protein.

We have prepared a "standard cellulase enzyme" preparation for NREL's use and have begun the process of evaluating storage conditions (i.e., -20°C). We suspect it will take several months to confirm that activity of the enzyme preparation is not lost during storage. As it stands now, there does not appear to be a significant loss of activity due to freezing. Once confirmed, these samples will be distributed throughout NREL for researchers conducting FPU assays.

NREL researchers have determined that a 90:9:1 mixture of an exoglucanase from *T. reesei* (CBH I), a thermal tolerant endoglucanase from *A. cellulolyticus* (EI), and a -D-glucosidase was capable of nearing the performance (i.e., end-point saccharification at 120 hours of cellulose in PYP) observed for a leading commercial *T. reesei* preparation at comparable protein loading. This discovery was an important breakthrough, because the option to engineer cellulase systems for specific biomass feedstocks became apparent for the first time. Work at NREL in FY1997 to 1998, with collaboration from the University of Arkansas (J. Sakon), presented another breakthrough in the cellulase biochemistry field. We showed that the performance (measured as maximum final percent saccharification of biomass) of this ternary system was improved by 12%, following site-directed modification of one active site amino acid residue identified from a high resolution X-ray crystallographic structure of EI. We are now pursuing another promising class of mutations for EI: those that modify the chemistry of the biomass interactive surface. We are now in the process of building new coding sequences that provide amino acid substitutions designed to alter the strength of binding the enzyme to cellulose. These sites are defined as "EI classes I & II" and replace amino acids capable of displaying hydrogen bonding and/or Van der Waal's interactions. The unproven theorem is that various combinations of these mutations could alter enzyme/substrate residence time and enhance specific activity.

## General Technical/Scientific Progress

### Site-Directed-Mutagenesis of *T. reesei* CBH I

*Trichoderma reesei* CBH I is a mesophilic cellulase that plays a major role in the hydrolysis of cellulose. To enzymatically hydrolyze biomass more effectively; it is essential that key thermophilic cellulases are developed or discovered. We have chosen site-directed-mutagenesis preliminarily as the tool for improving the thermal tolerance of *T. reesei* CBH I. Our ultimate biochemical objective is to improve the half-life of CBH I from half a day to 5 days at 60°C as measured by dialysis saccharification assay, or demonstrate a 10°C increase in the structural stability of rCBH I measured by differential scanning calorimetry.

We are proceeding very well in the production of reduced glycosylation *T. reesei* CBH I mutations *A. awamori*, thanks to the application of a new affinity column matrix designed for cellobiohydrolases. We have prepared 2-8 mg quantities each of several of the single, double, and triple N>A mutation of CBH I for subsequent biochemical analysis. Size exclusion chromatography of the concentrated affinity elution fractions shows well-behaved symmetrical peaks, much improved over the protocol we were using previously. We have recently improved all aspects of studying CBH I expressed in *A. awamori*, including a more efficient procedure for selection of transformed clones, improved fermentations for higher biomass production, and an improved purification procedure. This new series of cleaner, more active *T. reesei* CBH I expressed in *A. awamori* will be a better test platform for the specific site mutations we have identified, which should confer enhanced thermal tolerance.

## Scientific Publications, Posters, Presentations, and Other Activities

### General Presentations

W.S. Adney, J.O. Baker, S.R. Decker, S.L. McCarter, J. Sheehan, T.B. Vinzant, and M.E. Himmel (2000) "Improved Cellulases for Bioethanol Production," invited presentation at Wood and Cellulose: Building Blocks for Chemicals, Fuels, and Advanced Materials, SUNY College of Environmental Science & Forestry, Syracuse, NY, April 9-11, 2000.

S.L. McCarter, W.S. Adney, J.O. Baker, T.B. Vinzant, R.D. Guckian, S.R. Decker, J. Sakon, and M.E. Himmel (2000). "Site-Directed Mutagenesis of the EI Endoglucanase from *Acidothermus cellulolyticus*" Accepted for presentation at The 22nd Symposium on Biotechnology for Fuels and Chemicals, Gatlinburg, TN, May 2000.

S.R. Decker, W.S. Adney, J.O. Baker, S.L. McCarter, T.B. Vinzant, J. Sakon, K.L. Barnett, and M.E. Himmel (2000) "Heterologous Expression of *Trichoderma reesei* CBH I: Effect of Site-Directed Mutations on Expression and Thermostability," Accepted for presentation at The 22nd Symposium on Biotechnology for Fuels and Chemicals, Gatlinburg, TN, May 2000.

### **The following abstracts were submitted for consideration:**

W.S. Adney, M.E. Himmel, I. Andreeva, N. Pechurkina, G. Karpov, and V. Repin (2000). "Thermophilic Bacteria of the Kamchatka Peninsula as Producers of Carbohydrate Degrading Enzymes," Combined Rocky Mountain and Pacific ACS Regional Meeting, Idaho Falls, Idaho, June 14, 2000.

### **Scientific Journals and Books**

J. Baker, M.R. King, W.S. Adney, S.R. Decker, T.B. Vinzant, S.L. Lantz, R.E. Nieves, S.R. Thomas, L.-C. Li, D.J. Cosgrove, and M.E. Himmel (1999). "Investigation of the Cell Wall Loosening Protein Expansion as a Possible Additive in the Enzymatic Saccharification of Lignocellulosic Biomass," Applied Biochemistry and Biotechnology, 1999. In Press.

S.R. Decker, W.S. Adney, T.B. Vinzant, and M.E. Himmel (2000) "Two Novel Alkalotolerant Dextranases from *Streptomyces anulatus*," In *Glycosyl Hydrolases for Biomass Conversion*, (M. Himmel, J.O. Baker, and J. Saddler, eds.), ACS Series 769, American Chemical Society: Washington, DC, 2000. In press.

R. Palma, M.E. Himmel, G. Liang, and J. Brady (2000). "Molecular Mechanics Studies of Cellulases," In *Glycosyl Hydrolases for Biomass Conversion*, (M.E. Himmel, J.O. Baker, and J. Saddler, eds.), ACS Series 769, American Chemical Society: Washington, DC, 2000. In press.

R Palma, M.E. Himmel, and J. Brady (2000). "Calculation of the Potential of Mean Force for the Binding of Glucose to Benzene in Aqueous Solution", *J. Phys. Chem.* 2000, Submitted.

M.E. Himmel, J.O. Baker, and J. Saddler, eds. (2000) "Glycosyl Hydrolases for Biomass Conversion," ACS Series 769. American Chemical Society: Washington, DC, 2000. In press.

### **Technology Transfer**

M.E Himmel was invited to give a presentation entitled "Improved Cellulases for Bioethanol Production," at the Biotechnology Center, University of Utah, Logan, UT, March 2000.

M.E. Himmel attended a Gordon Research Conferences planning workshop for Chairs and Vice-chairs held in Los Angeles, February 2000.

M.E. Himmel and J. Baker co-edited (with J. Saddler, Univ. B.C.) a new ACS Book entitled "Glycosyl Hydrolases for Biomass Conversion." This book package is now complete and will be available as ACS volume 769 in about 4 months. The book features 14 chapters from internationally recognized researchers in the field as well as a special introductory chapter written by J. Sheehan which reviews future prospects/opportunities/and risks for bioenergy in the U.S.

## **Project Operation**

The university subcontracts supporting cellulase research at NREL and planned for FY00 (University of California at Davis, Cornell University, and the University of Arkansas) have been placed on schedule and by a competitive process.

S/C: Provide High Resolution X-ray Structures. The subcontractor at the University of Arkansas (Joshua Sakon) worked to support site-directed-mutation studies of *Acidothermus* EI and *T. reesei* CBH I. Crystals of CBH Icd were obtained and are ready for diffraction. One key EI mutant has also been crystallized.

S/C: Develop and Test Improved *T. fusca* Cellulases. The subcontractor at Cornell University, D. Wilson, has worked to *improve* *T. fusca* E3 and E5 by site directed mutagenesis. Wilson's lab is also evaluating new cellulase mixtures and cellulose surface binding phenomena.

S/C: Model Cellulase/Cellulose Interactions. The subcontractor at Cornell University, John Brady, has worked to apply molecular mechanics modeling to enhance our understanding of cellulase action. This information is being used to design protein engineering experiments at NREL and elsewhere.

## **Total Quality Management and ES&H**

The ET team received the DOE Diversity Stand-down and NREL Waste Minimization training.

## STRAIN DEVELOPMENT

### Summary of Technical Achievements or Results

A formal Cooperative Research and Development Agreement (CRADA) contract was signed between NREL and Arkenol Holdings of Mission Viejo, California to develop a genetically altered microorganism capable of rapid and efficient fermentation of sugars derived from rice straw biomass to ethanol. The CRADA was set up after NREL identified a strain of *Zymomonas mobilis* that had superior fermentation performance in Arkenol hydrolysates. The 1-year agreement officially started on August 1, 1999.

The objective of this CRADA will be to develop superior strains of a patented bacterium that could lead to faster and more efficient fermentation of rice straw to ethanol. The *Z. mobilis* strain, which has been identified as a good candidate for Arkenol's process, will be genetically manipulated to create a stable strain capable of fermenting both six-carbon and five-carbon sugars. In addition to the genetic work, NREL will utilize its Process Development Unit (PDU) facility to generate large-scale fermentation data and materials useful for Arkenol in acquiring engineering guarantees for their planned facility in Sacramento. We will conduct several experiments to show the stability of the microorganism and the repeatability of the process.

Arkenol requested that we conduct a five-stage fill-and-draw fermentation at a minimum scale of 150-liter fermentation vessels. A fill-and-draw fermentation is a batch fermentation in which the majority of the culture is drawn from the tank when fermentation is complete. That volume is replaced with fresh media (or hydrolysate) and restarted using the portion of the previous culture (left in the tank) as the starting inoculum for the second batch. Arkenol requested that the fill-and-draw operation be repeated for five fermentations to determine the consistency and stability of the organism. They also asked that we repeat the entire five-stage run in triplicate to verify the repeatability of the fermentation process.

The fermentations were conducted using a plasmid-bearing *Zymomonas mobilis* strain that is capable of fermenting xylose and has shown to be tolerant to high sugar feedstocks. Arkenol does not have the capability to supply the quantities of hydrolysate needed for this scale of operation, so a synthetic form of hydrolysate was used for these experiments. First we modified the vessels in order to obtain accurate carbon dioxide measurement and approval by the Institutional Biosafety Committee to use recombinant *Zymomonas* in the pilot plant. Once that was completed we conducted the fermentations.

Two runs were completed and showed very consistent and repeatable results. Final ethanol process yields for the five stages averaged 90.6%  $\pm$  1.6% in the first run. For the second run, the average ethanol process yield was 89.1%  $\pm$  0.9%. Arkenol was so pleased with the results from the duplicate runs that they did not request a third replicate be conducted.

## General Technical/Scientific Progress

### Development of Genomic Integrated Xylose-Fermenting *Z. mobilis* Strain for High Sugar Fermentation

Two gene integration systems are being evaluated to determine which system effectively integrates xylose-fermenting genes into the chromosomal deoxyribonucleic acid (DNA) of *Zymomonas mobilis* ATCC31821. We have started using homologous recombination methodology but no integrants were formed using the *ldh* gene constructed from *Z. mobilis* ATCC39676 with a simple tetracycline marker. A longer fragment of the *ldh* gene was created by PCR from 31821 and cloned into an appropriate vector containing a tetracycline (*Tc*) marker. An additional construct was made with the same *ldh* fragment flanking transaldolase/transketolase genes (half of the genes required for xylose fermenting capabilities) along with a *Tc* marker. After the genes are inserted into the cell, they are repeatedly cultured under selective conditions to allow recombination of genes to occur. During repeated transfers, the selection pressure is removed to select for nonplasmid-bearing strains. After several weeks of transferring and plating on selective agar, we identified several potential isolates. A PCR screening experiment was conducted to determine the size of the *ldh* gene in the selected isolates.

Ten isolates from each construct were evaluated and each produced a PCR product the size of the *ldh* fragment plus the additional *Tc* and *Tc/tal/tkt* genes. Southern hybridization indicated that the genes were integrated in the total genomic DNA. Further examination will be conducted to determine if the transaldolase and transketolase are functional.

### Evaluation Of Fermentation Performance Of Integrated Xylose- And Arabinose-Fermenting *Z. Mobilis* Strains In Continuous Fermentation

Two genomic integrated xylose and arabinose-fermenting *Zymomonas* strains developed through both homologous recombination and transposition, AX101 and G8, and plasmid-bearing strain 206C(pZB301) were evaluated for growth profile on pure sugar RMGXA (40:40:20 g/L for glucose, xylose, and arabinose) at pH 5 and 5.5. At both pH levels, strain AX101 performed slightly better than the other two strains. At pH 5.5 the ethanol process yield was 83%, 84%, and 76% for plasmid-bearing strain, AX101 and G8 respectively. At pH 5 the ethanol yield was 83%, 83%, and 67% respectively.

Sugar utilization by strain AX101 was better than the other two strains at both pH levels. Both integrated strains had higher xylitol production than strain 206C(pZB301). Based on these results, strain AX101 was selected for further evaluation in continuous fermentation.

A continuous fermentation using strain AX101 was started at pH 5.5 and a temperature of 30°C. The media used was RMGXA (40:40:20 g/L). The starting dilution rate for both was 0.02 (1/h) equivalent to 50 hours residence time. The dilution rate was increased gradually after five cycles of steady state. At  $D = 0.04$  (1/h) (25 hours residence time) and the remaining sugar in the fermenter was 0, 1.6, and 5.6 g/L for glucose, xylose, and arabinose, respectively, and the ethanol yield was 94%. After 28 days the pH was lowered to 5 and dilution rate was lowered to 0.027(1/h). The fermentation was continued for another 21 days at which time the dilution rate was 0.033 (1/h) and remaining sugar was 0, 1, and 6 g/L glucose, xylose, and arabinose, respectively, and ethanol process yield was 90%. At this time the pH was lowered further to 4.5 and fermentation was continued at dilution rate of 0.022 (1/h), then increased to 0.028 (1/h). The fermentation at pH 4.5 was stopped after 20 days, at which time the remaining sugar and ethanol yield was similar to pH 5 results as mentioned above.

### Construction of a DLDH- and LLDH-Negative *Lactobacillus* MONT4 Derivative

The construction of a DLDH- and LLDH-negative *L. MONT4* derivative utilizing multiple experimental approaches has been a quite challenging process. In order to better understanding the physiology of *L. MONT4* we have conducted experiments in which we followed growth, acid production, glucose

utilization, lactic acid production, and ethanol production at several growth temperatures (37 °C, 42 °C and 45 °C) under both aerobic and anaerobic conditions. The specific growth rates of *L. MONT4* at 37 °C, 42 °C, or 45 °C under anaerobic conditions are very similar. However, *L. MONT4* has the shortest lag period at 42 °C, followed by 37 °C and 45 °C. The same general pattern followed under aerobic conditions but the strain grew much slower and had much longer lag phase. The final cell densities obtained for *L. MONT4* grown at any growth temperature under anaerobic conditions are significantly higher than those obtained for *L. MONT4* grown at any growth temperature under aerobic conditions. The profiles of acid production, glucose utilization, and lactic acid production were tightly associated with growth. *L. MONT4* does not produce any ethanol at any growth temperature tested under either aerobic or anaerobic conditions. These results suggested that *L. MONT4* does not have an obvious alternative pyruvate-utilizing pathway.

We also started to clone the putative promoter of the LLDH from *L. MONT4*. Previously, the LLDH structural gene from *L. MONT4* was cloned in *E. coli* DH5a using degenerate primers based on sequenced LLDH genes from lactic acid bacteria. The objective of this experiment is to clone the complete LLDH gene from *L. MONT4*, including the gene expression signals. We believe that the putative constitutive LLDH promoter is a strong promoter and can be placed in front of the *pdc adh* operon to enhance expression levels. The chromosomal DNA from *L. MONT4* was digested with a variety of restriction enzymes. Southern hybridizations were performed using the digested chromosomal DNA preparations and a 785-bp LLDH probe. The chromosomal regions that hybridized to the LLDH probe were determined. We used this information to map both upstream and downstream regions of the chromosomal LLDH gene. Based on the newly constructed map of the LLDH gene, we selected several restriction enzymes as the cloning enzymes. We have identified several positive clones and the DNA will be sequenced.

### **Selection Of Second Generation Microorganisms For Ethanol Production From Lignocellulosic Biomass**

*Lactobacillus* is capable of fermenting many of the sugars commonly found in biomass and offers potential advantages in biomass-to-ethanol fermentations, including high ethanol tolerance, resistance to inhibitors present in hydrolysates, fermentation at low pH, and thermotolerance. *Lactobacillus* was identified as a promising genus of microorganisms for metabolic engineering for ethanol production in our previous study. In particular, a unique *Lactobacillus* strain, *MONT4*, capable of growth up to 45°C, is a promising microorganism for metabolic engineering for ethanol production from renewable lignocellulosic biomass. This is because of *MONT4*'s broad substrate fermentation range and its unique ability to metabolize pentose sugars, such as L-arabinose and D-ribose through a homofermentative pathway.

Previous study has shown that *L. MONT4* can tolerate 80% (v/v) overlimed poplar wood hydrolysate and 8% (v/v) ethanol at 37 °C, respectively. To access the capability of the potential ethanologen, *L. MONT4*, we conducted a study using factorial design to evaluate the inhibition of cell-growth and glucose conversion by *L. MONT4* in the presence of overlimed hardwood hydrolysate and exogenous ethanol. Cell growth and sugar conversion by fermentation to lactic acid was determined by starting cultures at low cell densities ( $A_{600}=0.25$ ) and at intermediate cell densities ( $A_{600}=2.0$ ). The pH was moderated in selected cultures for evaluating glucose conversion by adding finely powdered  $\text{CaCO}_3$ .

Growth is more sensitive to all combinations of inhibitors than is glucose conversion. The effects are more pronounced at the higher temperatures. We found that 5 % (w/v) ethanol appears to be more toxic than 80% (v/v) hydrolysate in cultures without pH moderation.

Moderating the culture pH above 5.0 significantly reduces inhibition for all conditions, especially at higher temperatures. The pH moderation reduces inhibition of both hydrolysate and

hydrolysate/ethanol mixtures, as expected. Interestingly, pH moderation also reduces inhibition by ethanol alone. All conversions were increased to 100 %, except at the highest concentrations of both hydrolysate and ethanol at the higher temperatures (80% for 41°C and 30% for 45°C).

The above results demonstrated that L. MONT4 can well tolerate 5 % (w/v) ethanol and overlimed 80% (v/v) hydrolysate with moderating pH above 5 at 41 °C.

### **Development of Arabinose-Fermenting Yeast**

This project is a funds-in CRADA with CRA/NCGA. We previously reported that we cloned three arabinose-assimilating genes *araA*, *araB*, and *araD* in TRP1 vector and transformed these plasmids to an appropriate yeast strain, respectively. The transformants were isolated and assayed for the enzyme activities. The L-ribulokinase (*araB*) and L-ribulose-5-P-4-epimerase (*araD*) were expressed at high levels and L-arabinose isomerase (*araA*) was expressed at a low level. We have since constructed two sets of *araA*, *araB*, and *araD* expression plasmids in the HIS3 and URA3 yeast vectors. We also constructed a set of seven yeast strains that carry the expression plasmids for each of the *ara* genes with all possible combinations of the yeast selection markers (TRP1, HIS3, and URA3). In addition, we constructed the control strain that carries the three empty vectors. The transformed strains were assayed for the three enzyme activities. Growth tests with these strains revealed that none of them grew on L-arabinose. We have conducted cell-free fermentations with the strains carrying the three *ara* expression plasmids and the control transformants. The extracts from cells grown under the most favorable conditions convert some arabinose to ethanol after prolonged incubation, whereas the control strain does not produce any ethanol under the same conditions. This result is very encouraging. We are now further characterizing these strains for *in vivo* fermentation.

### **Scientific Publications, Presentations, and Other Activities**

#### **Patents or ROI Filed**

M. Zhang and YC Chou filed two ROIs on “stable xylose and arabinose-fermenting *Zymomonas* strains” and “Method for gene inactivation in *Zymomonas*”

#### **Project Operation**

The SDT held two CRADA review meetings with the CRA/NCGA on January 7 and March 31, 2000. We provided the technical progress to the technical panel for both CRADAs (*Zymomonas* and yeast). Technical progress was presented to the representatives from Cargill, A.E. Staley, ADM, and CRA. We have met all the CRADA research objectives. The feedback received from CRA is very positive.

#### **Publications, Posters, and Presentations at Scientific Meetings**

An abstract entitled “Genetic improvement of *Zymomonas mobilis* for ethanol production: chromosomal integration of the xylose and arabinose-Fermenting genes” by M. Zhang, Y.C. Chou, A. Mohagheghi, K. Evans, S. Milstrey, X.K. Lai and M. Finkelstein was submitted for oral presentation at the 22nd Symposium on Biotechnology for Fuel and Chemicals in Gatlinburg, TN, May 6-11, 2000.

An abstract entitled “Cofermentation of glucose and xylose by integrated strains of *Zymomonas mobilis*” by K. Evans, A. Mohagheghi, Y.C. Chou, and M. Zhang was submitted for poster presentation at the 22nd Symposium on Biotechnology for Fuel and Chemicals in Gatlinburg, TN, May 6-11, 2000.

An abstract entitled “Evaluation of *Lactobacillus* MONT4 for cell-growth and glucose conversion in the



presence of overlimed hardwood hydrolysate and ethanol” by A. Mohagheghi, F. A. Keller, and M. Zhang was submitted for poster presentation at the 22nd Symposium on Biotechnology for Fuel and Chemicals in Gatlinburg, TN, May 6-11, 2000.

## ENZYMATIC PROCESS DEVELOPMENT

### Summary of Technical Achievements or Results

The Enzymatic Process Development (EPD) team achieved several important technical accomplishments:

- Two milestones to develop and validate rapid analysis methods based on NIR spectroscopy were successfully met on schedule during the reporting period.
  - In January, we completed development of a NIR spectroscopy-based rapid analysis method for the compositional analysis of hybrid poplar feedstocks and dilute acid pretreated poplar sawdust solids (process intermediates). Two NIR-based methods were developed, one for raw yellow poplar feedstock and one for pretreated yellow poplar. The precision and accuracy of these two methods match those of the conventional wet chemical techniques used to analyze the compositions of the calibration samples. The fully cross-validated feedstock method predicts the percentages of extractives, lignin, glucan, and xylan with a root mean square error of prediction (RMSEP) of approximately 2% for each constituent. The initial fully cross-validated method for pretreated feedstock predicted the percentages of lignin, glucan, and xylan with an approximate RMSEP of less than 2.5%, which was considered sufficient for application of the method to begin to expedite countercurrent hydrolysis reactor testing. We have since further improved the xylan correlation, increasing the correlation coefficient from 0.704 to 0.905, by incorporating additional calibration samples as they have become available from the countercurrent reactor evaluation work. The improved pretreated feedstock or process intermediates method has a wider dynamic range and continues to be used to hasten countercurrent pretreatment reactor performance.
  - In March, we successfully developed a NIR-based rapid analysis method for quantifying the proportions of *Trichoderma reesei* fungal cell mass and cellulose (as Solka-floc, a purified cellulose substrate). This method is being used in conjunction with total solids concentration measurements to quantify cell mass and residual cellulose concentrations in the cellulase production broth samples. Validation testing shows that models based on two of the three calibration sets that were tested (the weighed and combined sets discussed below) are capable of predicting cell mass concentrations with a standard error of less than 8%. This work was written up in a March P-milestone report. Cell mass concentration and cellulose concentration data obtained using these predictive models are currently being used to assess carbon balance closures on enzyme production as well as to estimate kinetic model parameters for fungal growth and enzyme production.
- Corn stover process development activities were initiated. Emphasis during the reporting period was on improving analytical methods for corn stover compositional analysis and readying the pilot scale feed handling and pretreatment reactor systems for exploratory pretreatment work. EPD team members also assisted in completing an initial round of exploratory corn stover pretreatments in the minipilot scale steam gun pretreatment reactor.
- We increased our outreach efforts to the cellulase enzyme production industry, sending letters describing EPD's planned R&D activities related to cellulase enzyme production and use to Genencor International, Novo Nordisk Biotech, and Iogen Corporation. These letters request comments from industry on our work plans and should help to ensure that our work in these areas is supporting and adding value to industry's nascent efforts to commercialize commodity cellulase production.

## General Technical/Scientific Progress

EPD team activities are focussed on core technology development. Our objective remains to develop integrated enzymatic cellulose hydrolysis-based process technology that is capable of achieving the MYTP year 2000 bioethanol process performance goals. While we are now transitioning from yellow poplar sawdust to corn stover as our primary biomass feedstock, our process development efforts otherwise remain similar to those described for the last reporting period. In particular, the base-case enzymatic cellulose hydrolysis-based process that we are developing and demonstrating continues to be based upon co-current dilute sulfuric acid prehydrolysis or pretreatment to solubilize the hemicellulosic sugars and increase the enzymatic digestibility of the cellulose. And cellulose conversion is still being carried out simultaneously with mixed biomass sugar ethanol fermentation using a SSCF process configuration. We have initiated work to explore the potential of ethanologens beyond *Zymomonas mobilis*, but for the time being are continuing to use an adapted variant of xylose-fermenting *Z. mobilis* as the SSCF microorganism. Cellulase enzyme continues to be made by the fungus *Trichoderma reesei* using cellulose as the primary carbon source.

The team's major thrusts over the past 6 months were to develop rapid methods for compositional analysis of yellow poplar hardwood feedstock and process intermediates and to initiate corn stover process development. Corn stover-related work included: improving conventional compositional analysis methods for corn stover samples; continuing the long-term corn stover compositional stability study; making preparations to carry out continuous pilot scale pretreatment of corn stover; and assisting the IPD team with exploratory batch stream gun pretreatments of corn stover. Secondary thrusts during the reporting period were in the areas of cellulase enzyme production, cellulase enzyme efficacy assessment, integrated SSCF performance benchmarking, and SSCF fermentative strain evaluation.

## Development and Application of Rapid Analysis Methods

Rapid analysis methods offer the promise of greatly reducing both the time and cost of performing biomass compositional analysis. As a consequence of substantial instrumentation and labor requirements, biomass compositional analysis is typically an expensive bottleneck in R&D. Recognizing the benefits to improved research efficiency, the Ethanol Project has increased its emphasis on developing and applying rapid analysis methods that can be used to expedite integrated process development. Two important accomplishments that are related to further developing rapid analysis methods were achieved during the reporting period.

In January, we completed development of an NIR spectroscopy-based rapid analysis method for the compositional analysis of hybrid poplar feedstocks and dilute acid pretreated poplar sawdust solids (process intermediates). The approach was to build off our existing compositional data on yellow poplar feedstocks and pretreated solids. Two NIR-based methods were developed. The precision and accuracy of these two methods match those of the conventional wet chemical techniques used to analyze the compositions of the calibration samples. The feedstock sample set used to calibrate the NIR method for raw yellow poplar feedstock consists of 34 samples that encompass 17 different hardwood species. This fully cross-validated method predicts the percentages of extractives, lignin, glucan, and xylan with an RMSEP of approximately 2% for each constituent. Independent analysis of yellow poplar samples using this new NIR rapid analysis method thus provides chemical composition results that are in good agreement with those obtained using traditional wet chemical methods. The pretreated yellow poplar solids calibration set consists of 44 samples generated over a wide range of dilute acid pretreatment conditions, i.e., from very mild pretreatment conditions achieving incomplete hemicellulose hydrolysis to much more severe pretreatment conditions that achieve nearly complete hydrolysis of both hemicellulose and cellulose. This fully cross-validated method predicts the

percentages of lignin, glucan, and xylan with an approximate RMSEP of less than 2.5%. The agreement between the "real" compositions determined by wet chemical methods and those predicted using the NIR method is excellent for lignin and glucan, which exhibit correlation coefficients of 0.963 and 0.975, respectively. However, the correlation coefficient for xylan is only 0.704. This poorer correlation for xylan reflects a high standard error in the wet chemical method used to quantify xylan relative to the xylan values (1.5% absolute error in values ranging from 0%-7%) as well as the need to add additional samples to the pretreated feedstock calibration set. This work was written up in a C-milestone report (#3). Since January, we have improved the xylan correlation, increased the correlation coefficient from 0.704 to 0.905, and increased the method's robustness and dynamic range by adding additional calibration samples as they become available (i.e., as the countercurrent reactor evaluation work proceeds). The improved process intermediates method is now being applied to facilitate performance testing of the countercurrent pretreatment reactor. Using this method is speeding determination of reactor efficiency and should help to expedite optimization of reactor performance for both partial and total hydrolysis applications.

In March, we successfully developed an NIR-based rapid analysis method for quantifying the proportions of *Trichoderma reesei* fungal cell mass and cellulose (as Solka-floc, a purified cellulose substrate). This method is useful for quantifying cell mass and residual cellulose concentrations in the heterogeneous mixed solids samples that are obtained after filtering cellulase production broths. It must be emphasized that measurements of cell mass concentration in the presence of substrate solids are essential to develop and validate reliable kinetic models for *T. reesei* cell growth and cellulase enzyme production. In this work, two different approaches were used to prepare a total of three different calibration data sets for the rapid analysis methods. A weighed calibration set was prepared by manually combining cell mass and Solk-floc. A DNA calibration set was obtained by estimating the cell mass content of actual production samples based on DNA analysis of the samples. The third set was a combination of both types of calibration samples. For each calibration set, projection to latent structures (PLS) analysis was used to develop a model for predicting the proportions of cell mass and cellulose from NIR spectra of mixed solids samples. Validation test results show that two of the models (weighed and combined) are sufficiently predictive to be used for kinetic modeling efforts; these two models are capable of predicting cell mass concentrations with a standard error of less than 8%. This work has been written up in a P-milestone report. Cell mass concentration and cellulose concentration data obtained indirectly using the predictive models are currently being used to estimate kinetic model parameters for fungal growth and enzyme production. The cell mass concentration and residual cellulose data are also being used to assess carbon balance closures on enzyme production. Ultimately, this NIR/PLS rapid analysis approach will be extended to measure cell mass concentrations in the presence of lignocellulosic substrates such as corn stover.

### **Corn Stover Process Development**

Our FY00 process development objective is to develop a baseline for integrated corn stover conversion performance. Initial efforts are focussing on refining our compositional analysis methods for corn stover (see below), which is chemically dissimilar to yellow poplar feedstock in several aspects. A parallel thrust during the reporting period has been readying the pilot plant's feedstock handling and Sunds reactor systems for carrying out corn stover pretreatment. To this end, we obtained approximately 350 kilograms (dry basis) of coarsely milled corn stover from the corn stover collection facility in Harlan, Iowa, early in the reporting period. This amount of material enabled us to refine our compositional analysis methods and shake down our feed handling systems prior to obtaining a larger batch of material to use for systematic pretreatment experiments.

### **Corn Stover Compositional Analysis**

Efforts during the reporting period initially focussed on improving the quality of traditional wet chemical

methods for determining corn stover composition. Work early in the reporting period showed poor overall mass balance closures on corn stover feedstock compositional analyses, with mass balances occasionally closing to only about 85%. In addition, unexpectedly high levels of ash were observed in many of the samples. Results ultimately showed that a significant amount of dirt was present in many of the samples, and that this was confounding both the wet chemical analysis and early NIR spectroscopic scans of these materials. We have since determined that washing the solids with water and/or milling and sieving them (and analyzing the +80 mesh fraction) can remove entrained dirt from the samples and thereby improve the reproducibility and reliability of sampling methods, compositional analysis, and NIR spectroscopy. The wet chemical methods that were used for the first round of analysis were based on techniques optimized for wood samples, which have no measurable protein component and typically have ash contents less than 1% (by weight, dry basis). In contrast, as received corn stover samples that have protein contents as high as 10% and ash contents in the range of 5%-30%. Thus, during the course of this work, the analytical methods optimized for wood were modified to improve their accuracy for corn stover samples. Implementing these minor changes to the analytical methodology has improved mass balance closures on analyzed corn stover feedstock solids by approximately 5%-10%, such that closures are now typically 90%-95%. Work to further improve our analytical methods for corn stover continues. In particular, we plan to eventually develop and validate additional analysis procedures to quantify uronic acids and minor hemicellulose components. The addition of these analyses is expected to bring routine mass balance closures on analyzed corn stover samples up to approximately 98%.

### **Corn Stover Compositional Stability Study**

The long-term corn stover compositional stability study continued during the reporting period. The focus was primarily on improving the analysis protocol, however, as described above. This is because small changes in composition such as are anticipated during long-term storage will be difficult to observe if the accuracy or variance of the underlying compositional analysis is poor. Towards the end of the reporting period, after an improved analysis protocol had been established, a third round of samples was obtained from Harlan. The same subset of these samples that has been analyzed in each of the first two rounds was worked up for compositional analysis. The findings of the first three rounds of sampling, which cover two different harvest years and 6 distinct corn stover bale ages, will be communicated in an April C-milestone report (#7).

### **Corn Stover Pretreatment**

**Sunds Reactor Pretreatment System.** Significant progress was made during the reporting period in troubleshooting and improving our pilot scale corn stover handling and pretreatment capabilities. In particular, the continuous pilot scale Sunds Hydrolyzer pretreatment reactor system was extensively repaired and modified. Significant repairs and modifications to the feed handling and pretreatment reactor system were also carried out. Some of the more critical repairs made to the system include:

- 1) installed, tested, and adjusted a new screw auger drive on the feed hopper (this is used to control the rate at which feedstock material is fed from the feed hopper);
- 2) updated the P&ID diagrams for the Sunds reactor and it's flash tank (this was necessary to finalize the lockout/tagout procedures prior to beginning repairs);
- 3) rebuilt the plug flow feeder;
- 4) installed new and fabricated parts for the vertical impregnator section of the Sunds reactor; and
- 5) removed the displacement cylinder from the Sunds reactor.

At the end of the reporting period the feed handling and pretreatment system had been completely rebuilt and shakedown testing with corn stover commenced. Initial shakedown testing of the rebuilt Sands hydrolyzer reactor system using corn stover (after a water wash and air drying) was successful, with the pug mill, cross feeder, and plug feeder all functioning well enough to enable steady feeding of the Sands reactor to be achieved. The refurbished Sands reactor system therefore appears capable of processing coarsely milled (and then washed and dried) corn stover reasonably well. Despite these encouraging results, a couple of minor issues were identified that need to be acted on before further testing can occur. First, a different squeeze pump better suited to handle the relatively fibrous corn stover squeeze slurry needs to be installed; a different pump that we have on site should work. Second, minor additional modifications must be made to the feed hopper and belt feeder system to improve their operability; these modifications are underway.

We will commence pilot scale pretreatment experimentation after we procure a large supply (approximately 5-10 tons) of corn stover from the Harlan site early in the next reporting period.

**Steam Gun Pretreatment System.** During the reporting period EPD members participated in completing a series of exploratory pretreatments of water-washed Harlan corn stover using the 4-L steam gun or steam digester. This work, which is being led by the IPD team, is intended to guide EPD's upcoming efforts to carry out pilot scale continuous pretreatment of corn stover in the Sands Hydrolyzer pretreatment reactor. One of the purposes of these experiments was to identify – at least at a preliminary level – dilute acid pretreatment operating conditions (residence time, reactor temperature, acid concentration) that can be used in the Sands to produce reasonably high yields of soluble xylose and enzymatically digestible cellulosic solids. This work was completed late in the reporting period, and chemical analyses of the pretreated corn stover samples must still be performed. Once results have been analyzed, follow-up steam experiments may be designed to better characterize the scaling relationship between the Sands and steam gun reactors. In addition, follow up experiments may be run to establish more efficacious conditions for the steam gun pretreatment.

### **Cellulase Enzyme Production**

Early in the reporting period, we successfully completed a P-milestone to investigate oxygen transfer capabilities in the Process Development Unit's (PDU's) 9000-L fermentors. Sufficient oxygen transfer is critical to achieving good enzyme production but oxygen transfer capabilities typically decrease with scale. Thus, it is important to understand the capabilities of the different sized vessels available for enzyme production, which range from bench scale fermentors (7-L vessels) to PDU seed fermentors (vessels sizes of 160-L and 1500-L) and production fermentors (9000-L vessels). Oxygen transfer capabilities in a 9000-L fermentor were assessed by measuring the oxygen uptake rate (OUR) during growth of *T. reesei* on a 5 % (w/w) glucose. Results demonstrated that, surprisingly, OURs of similar magnitude to those achieved during bench scale cultivation of *T. reesei* cultivation (7-L fermentors) can be achieved in the larger PDU fermentors (9000-L fermentors). This suggests that scale up of high productivity cellulase production protocols identified at the bench scale to the larger PDU vessels may not be as much of a problem as previously anticipated.

Other cellulase production activities were directed at establishing and validating methodologies for performing carbon mass balances on the enzyme production process. This work is moving forward and will be communicated during the next reporting period when a C-milestone documenting carbon balance closure is due.

During this reporting period the EPD team significantly increased its outreach efforts to the cellulase enzyme production industry. Letters and supporting information describing EPD's plans in the areas of cellulase enzyme process development were sent to representatives of Genencor International (M.

Knauf and V. Lentner), Novo Nordisk Biotech (J. Cherry), and Iogen Corporation (J. Tolan). These letters describe EPD's planned future work activities to produce cellulase enzymes on and apply cellulase enzymes to pretreated lignocellulosic substrates. Our objective is to solicit comments from companies currently producing commercial cellulases. This should help to ensure that our work in these areas is adding value to industry's nascent efforts to commercialize commodity cellulase production technologies. EPD's ultimate objective is to align our work to best support and facilitate the emergence of a low-cost commercial cellulase enzyme and otherwise facilitate the emergence of a domestic biomass conversion industry.

### **Cellulase Enzyme Assessment**

In advance of having pretreated corn stover feedstock to work with, we continued assessing enzyme quality and integrated SSCF performance using pretreated yellow poplar feedstock. As reported in the previous reporting period, the efficacy of *T. reesei* cellulase enzymes produced using cellulosic feedstocks appears to be significantly higher than the efficacy of the single (and relatively old) commercial cellulase preparation we have been using as a reference preparation; this preparation was presumably produced using soluble sugar(s). In particular, high levels of cellulose conversion are achievable in simultaneous saccharification and fermentation (SSF) and SSCF at much lower enzyme loadings using the cellulase enzymes produced using cellulose (Solka-floc). These observations suggest (but do not prove) that higher efficacy cellulase enzymes are produced when the cellulase-producing fungus is induced in the presence of cellulose. It is critical to test this hypothesis, however, because if it is true the soluble sugar-based enzyme production technologies that we believe are currently used to produce most commercial cellulases may be inappropriate for producing cellulases exhibiting high efficacy for complete cellulose saccharification applications. As part of an effort to better understand the generality of these findings, we carried out a series of additional SSF experiments during this reporting period to determine if other commercial cellulase preparations, i.e., those recommended by enzyme producers for complete cellulose saccharification applications, behaved similarly to the single commercial preparation we've used to date. To this end, EPD obtained samples of reputedly high-efficacy commercially available cellulase enzyme preparations from V. Lentner of Genencor International, J. Cherry of Novo Nordisk Biotech, and J. Tolan of Iogen. Efficacy testing of these additional cellulase preparations has commenced. Preliminary results support our initial findings, with the cellulose-grown cellulase preparation produced in-house outperforming to varying degrees all of the commercial preparations. Work to wrap up these studies is underway and will be reported in the next performance period. Results of this work will be communicated to industry as they become available, and will be used in conjunction with industry input to guide EPD's future applied enzyme production and usage efforts.

### **Integrated SSCF Performance Benchmarking**

In addition to cellulase enzyme quality assessments, we have completed several follow up experiments to replicate the significantly improved integrated SSCF baseline reported in FY99 September C-milestone (#26). These integrated SSCFs were rigorously sampled in order to enable carbon mass balance closures to be assessed. The objectives were twofold. First, we wanted to confirm that integrated SSCF performance levels indeed approach the year 2000 MYTP targets as suggested by our recent milestone work. Second, we want to generate a higher quality SSCF performance data set that can be used by the Process Engineering team to update the base-case ASPEN Plus-based technoeconomic model for an enzymatic bioethanol process. Work completed during the reporting period confirmed that greater than 80% conversion of cellulose (in the form of pretreated yellow poplar sawdust) can be achieved using a cellulase enzyme loading less than 15 FPU/g cellulose. In particular, our best-to-date integrated SSCF performance results show that 85% cellulose conversion can be achieved using a cellulase loading of 12 FPU/g cellulose (*T. reesei* enzyme produced using a cellulosic substrate). This 7-day SSCF converted 96% of the input xylose to fermentation products and achieved

an ethanol process yield (based on all available monomeric sugars) of 70% of theoretical; the ethanol yield from just xylose and glucose, the only sugars fermented by the *Z. mobilis* we are using, was 88% of theoretical. These data are internally consistent, with the carbon balance for this SSCF closing to 95%. In addition to this experiment, we completed several other integrated SSCFs to better characterize the dependence of performance on enzyme loading. These additional experiments were carried out at enzyme loadings ranging from 6 to 26 FPU/g. The results of this work suggest that cellulose conversion levels of 80% can be achieved using enzyme loadings as low as 10 FPU/g.

### **SSCF Fermentative Strain Evaluation**

The shift of the enzymatic cellulose hydrolysis process development effort from yellow poplar hardwood sawdust to corn stover agricultural residues presents an auspicious opportunity to reassess which fermentative microorganism should be used in the SSCF unit operation. We want to utilize the best available fermentative strain in our nascent corn stover process development effort. Work with yellow poplar feedstock has so far utilized an adapted variant of xylose-fermenting *Z. mobilis* strain 39676 (pZB4L) developed several years ago. While this strain has proven reasonably effective in integrated SSCF performance testing, it nonetheless suffers from two limitations. First, its substrate range is narrow. This strain ferments only glucose and xylose but not the other biomass sugars galactose, mannose, and arabinose; mannose is an important trace component in poplar and arabinose is an important trace component in corn stover. Second, the genes encoding for xylose utilization and xylose fermentation capability in this strain are borne on a plasmid that contains an antibiotic resistance marker; potentially mobilizable plasmid DNA, and especially antibiotic resistance genes are undesirable in a production microorganism. Thus, while *Z. mobilis* strain 39676 (pZB4L) has performed well and has been useful for establishing an integrated SSCF performance baseline, our longer-term goal is to utilize a strain that has a broader substrate range than just glucose and xylose and that does not contain antibiotic resistance genes.

For these reasons, we began evaluating some of the other cofermenting strains that are available to us to better understand their potential as alternative process strains. During the reporting period we obtained and began testing several new strains. These included several improved *Z. mobilis* strains, developed by the Strain Development team at NREL, that exhibit expanded substrate ranges. These strains can utilize arabinose in addition to glucose and xylose, and in some of the strains the foreign genes are integrated into the chromosome. We have also obtained access, through BC International (BCI), to two broad substrate range enteric ethanologenic strains developed by L. Ingram at the University of Florida at Gainesville. (BCI holds exclusive license to these strains.) These latter strains, which still contain gene encoding for antibiotic resistance, are capable of fermenting all of the biomass sugars and one can even ferment certain biomass sugar oligosaccharides. (We ultimately plan to obtain one of the better glucose-xylose cofermenting yeast strains developed by N. Ho at Purdue University.) We began comparative evaluations of these *Z. mobilis* and enteric strains in SSCF using pure substrates (mixtures of pure cellulose, xylose, and arabinose). Results are preliminary, but suggest that some of the alternative strains may enable improved SSCF performance to be achieved. In particular, preliminary work carried out using pure substrates suggests that one of the recently developed *Z. mobilis* strains, the chromosomal integrant strain AX101, may be competitive with strain 39676 (pZB4L). Further work will be conducted, as resources permit, to compare the performance levels of these two strains under more rigorously simulated SSCF processing conditions. We will also assess the comparative SSCF performance of other cofermenting strains identified as being promising in early screening tests. It should be noted that the enteric strains are being evaluated under the terms of the BCI CRADA and results for these strains will only be communicated after obtaining approval from BCI. All strains that consistently show comparable or better performance than our base-case *Z. mobilis* strain will eventually be tested on corn stover hydrolyzates under progressively more integrated and process relevant conditions in order to select the best strain for our initial corn stover process integration efforts.



## Scientific Publications, Presentations, and Other Activities

### Publications, Posters, and Presentations and Scientific Meetings

McMillan, J.D.; Dowe, N.; Mohagheghi, A.; Newman, M.M. (2000). "Assessing the Efficacy of Cellulase Enzyme Preparations under Simulated Processing Conditions." Invited chapter for forthcoming ACS book *Glucosyl Hydrolases for Biomass Conversion*, M. Himmel, ed. ACS Symposium Series 7, American Chemical Society: Washington, DC. In press.

McMillan, J.D. (1999). "Bioethanol: The NREL Experience." Invited lecture IX presented at the Sixth Seminar on Enzymatic Hydrolysis of Biomass, Maringá, Paraná, Brazil, December 6-10, 1999.

McMillan, J.D.; Dowe, N.; Farmer, J.D.; Hamilton, J.; Lyons, R.; Mohagheghi, A.; Newman, M.M.; Sáez, J.C.; Schell, D.J.; Templeton, D.W.; Tholudur, A. (1999). "Development of Integrated Enzyme-Based Process Technology for Ethanol Production from Biomass". Oral paper 30 presented at the Sixth Seminar on Enzymatic Hydrolysis of Biomass, Maringá, Paraná, Brazil, December 6-10 1999.

Newman, M.M.; Dowe, N.; Mohagheghi, A.; McMillan, J.D. (1999). "Decreasing the Enzyme Loading Required for Enzymatic Cellulose Conversion." Poster presentation by M. Newman at the Third Conference on Recent Advances in Fermentation Technology, November 13-16, 1999, Sarasota Springs, FL .

Ranatunga, T.D.; Jervis, J.; Helm, R.F.; McMillan, J.D.; Wooley, R.J. (2000). "Overliming of dilute acid pretreated lignocellulosics: The fate of inorganics, uronic acids and ether-soluble organics." *Enzyme Microb. Technol.* In press.

Tholudur, A.; Ramirez, W.F.; McMillan, J.D. (2000). "Interpolated Parameter Functions for Neural Network Models." *Comp. Chem. Eng.* In press.

Tholudur, A.; Hamilton, J.; Sáez, J.C.; Stanlis, K.; McMillan, J.D (1999). "Modeling Cellulase Production on Purified Cellulose Using *Trichoderma reesei* L-27." Oral presentation by A. Tholudur at the 1999 Annual Meeting of the American Institute of Chemical Engineers, October 31-November 5, 1999, Dallas, TX .

Tholudur, A.; Rameriz, W.F.; McMillan, J.D. (1999). "Mathematical Modeling and Optimization of Cellulase Production using *Trichoderma reesei* RL-P37." *Biotechnol. Bioeng.* 66: 1-16.

# **LIGNIN CONVERSION TO FUELS**

## **Summary of Technical Achievements or Results**

The goal of the Lignin Conversion to Fuels task is to develop processes for converting lignin into valuable fuel blending agents. Lignin is the biomass component with the highest energy content (9000 - 11000 Btu/lb vs. 7300 - 7500 Btu/lb for cellulose), and can be burned to provide process heat. The novel processes developed in this task are expected to increase the value of the lignin relative to its fuel value, thereby improving the overall economics of biomass to liquid fuels conversion technologies.

Given the molecular nature of lignin, it is conceivable to convert lignin into high-octane fuel additives. Specifications for gasoline set by the California Air Resources Board (CARB) and the Environmental Protection Agency (EPA) define the types of products to target. Gasoline can contain only low levels of benzene (<1%), a controlled amount of aromatics (<25%), and olefins (<5%), plus a small amount of oxygen (about 2%). The gasoline boiling point (90% should distill below 149 °C) and Reid vapor pressure (RVP) are also important parameters. RVP should be 48 – 56 kPa. The octane number determines gasoline fuel grade and ranges from 85 to 93. Valuable additives should therefore be hydrocarbons or oxygenates that have a high octane number (>110), and are compatible with the boiling point and RVP restrictions. From lignin it is possible to make aromatic hydrocarbons and/or ethers that fit within these specifications. This project is thus targeted to obtain the necessary scientific and technological data with which to evaluate the economics and markets for converting lignin into valuable fuel additives.

The National Renewable Energy Laboratory (NREL), the University of Utah (UU) and Sandia National Laboratory (SNL) are developing a base-catalyzed depolymerization (BCD) process that renders lignin more amenable to various hydrotreating strategies that can yield hydrocarbon or oxygenated fuel additives. UU researchers have shown that lignin can be effectively depolymerized using aqueous sodium hydroxide. However, base recovery can be an expensive process. Work is being conducted at all three laboratories to find cheaper base conditions for effectively depolymerizing lignin. UU has recently scaled up the BCD process from bench scale autoclaves to a flow reactor capable of processing at least 100 g/h of lignin.

UU is also investigating the hydrotreating process. Currently work is focussed on developing catalysts and process conditions for converting the BCD intermediate into a high-octane hydrocarbon fuel additive. Catalysts with hydrodeoxygenation and hydrocracking activities are being tested. Process conditions are being sought that allow use of these catalysts in a single step. Small samples of hydrocarbon products, that have been made from ethanol process lignins, have the desired characteristics for a high-octane fuel additive. Current goals for the process are to increase the amount of product in the gasoline boiling range and to limit production of low octane cyclohexanes.

UU has also been developing superacid catalysts that only partially deoxygenate the BCD intermediate to give low molecular weight phenolics. After etherification with methanol, methyl aryl ethers are made that could be good oxygenated high-octane fuel additives. UU has also developed catalysts that are effective for the etherification reaction.

## **General Technical/Scientific Progress**

At UU, testing of the BCD flow system has been ongoing during the past 6 months. Operation of the flow system has been gradually optimized. Deficiencies in the original design have been corrected and the malfunctioning of some system components, rectified. For example, the glass beads, initially used

as a packing material for the BCD tube reactor, were found to be easily corroded by hot aqueous NaOH solution. The beads now have been replaced by low porosity alkali-resistant pellets made from fused silica-alumina. This packing material is being used successfully in the current work to produce large BCD product samples. The BCD flow reactor is now fully operational and capable of producing 1-2 kg of depolymerized product per week, according to need.

During the reporting period, about 2 kg of extracted BCD product has been made in runs using the flow reactor under the following conditions. In 4 hours, lignin (0.5kg) dissolved in 3% NaOH solution, was processed through the BCD flow reactor operated at 320°C. The products were obtained, after acidification, by ether extraction of both the aqueous solubles (aqueous extract) and aqueous insolubles (soxhlet extract). The BCD lignin product is the sum of the two ether extracts. About 51% of the lignin was converted into the soxhlet extract and about 22% was converted to the aqueous extract. Ether insolubles amounted to about 21% of the lignin. The combined ether extract product (73% of the lignin) is currently being hydroprocessed to obtain a sufficiently large sample of the hydrocarbon product for testing its fuel properties.

Samples of the aqueous and soxhlet extracts from the flow reactor were provided to NREL by UU researchers for analysis. The soxhlet extract contained mostly methoxylated phenols with guaiacol and syringol being the largest volatile components. Methyl catechol was also a significant component in this sample. The aqueous extract contained a larger amount of low molecular weight phenolics, especially catechol (16% of the product) and other catechols. From the analyses it was estimated that 10% of the lignin was converted into identifiable volatile phenols; the majority of the product was higher molecular weight phenolic oligomers. The largest identifiable components in the combined BCD product were catechol, methyl catechol, methoxy catechol, guaiacol and syringol. The product is characteristic of a BCD made under relatively mild conditions. It should be suitable for making a hydrocarbon fuel additive; however, additional hydrogen will be needed to deoxygenate this BCD because of its relatively high oxygen content (25%).

SNL researchers have also been experimenting with lignin depolymerization catalysts, based on calcium and magnesium, which could be cheaply recovered by conversion to insoluble carbonates using carbon dioxide. The bases could then be regenerated thermally. The effectiveness of lignin depolymerization has been assessed at SNL by measuring the amount of insoluble product made. The performance of both hydroxides and oxides has been tested alone and in mixtures with sodium hydroxide. For both calcium and magnesium the oxides performed better than the hydroxides; however, none of them performed as well as sodium hydroxide alone. The most interesting results were obtained when sodium hydroxide (1%) was combined with the other bases. In almost every case the mixed base outperformed sodium hydroxide alone (46% insolubles at the 1% level). The best results were obtained with calcium oxide mixed with sodium hydroxide (25% insolubles).

SNL researchers recently supplied NREL with three samples of their BCD product made by accumulating material from 8 duplicate runs at 250°, 290°, and 330°C. The reaction mixture consisted of 1 part lignin, 9.2 parts water, 0.43 parts CaO, and 0.09 parts NaOH. The reaction products were acidified at Sandia, and worked up at NREL. Yields of BCD product increased with temperature from 10% at 250°C to 30% at 330°C. These yields are lower than previously obtained; however, the reaction times for these runs were only 5 minutes.

At NREL, all products from the three experiments were subjected to analysis using gas chromatography/mass spectrometry. The BCD products consisted mostly of methoxy phenols, including guaiacols and syringols. Syringol was the biggest component in all samples. Catechols were mostly produced at 330°C. Vanillin and syringaldehyde decreased as reaction temperature increased, as to a lesser extent did acetovanillone and acetosyringone. Generally, as the reaction temperature increased the complexity of the chromatograms increased. Also, products appeared at

the higher reaction temperature that were most likely generated from isomerization of the products made at lower temperature. Surprisingly, a number of methoxy benzenes were also produced, particularly 1,2,3-trimethoxy benzene. The methoxy benzenes tended to increase with increasing reaction temperature. Methoxy benzenes have been observed in some NREL and Utah aqueous BCDs, although not as much as appears to be present in the 330°C samples made at SNL. The products are characteristic of BCDs made under relatively mild conditions. They could be used to make a hydrocarbon fuel additive. Additional work must be performed, however, to increase the yields.

A report entitled "Review of Market for Octane Enhancers" by J.E. Sinor Consultants was recently completed. J.E. Sinor is a subcontractor to NREL who has significant experience in assessing the fuels market. The report details the specifications and properties a blending component must have and discusses cost and environmental considerations. Market and price projections are also reviewed.

In conclusion, the report states that there is continuing pressure on petroleum refiners to reduce the amount of aromatics in gasoline. However, it is expected that there will soon be new federal limits on sulfur. Meeting the sulfur caps will cause an unintentional reduction in aromatics and loss of octane number. The expected simultaneous phase-out of MTBE from the gasoline pool will create increased nation-wide demand for octane. This clearly opens the door for an aromatic blending agent to replace lost octane value by bringing the aromatics content back to allowable levels. The potential market volume under such a scenario could be on the order of 2 billion gallons per year. Because there is a clear need for a product to replace the octane that will be lost as a result of both desulfurization and MTBE phase-out, it is recommended that work continue in this area.

### **Scientific Publications, Presentations, and Other Activities**

An abstract entitled "Conversion of Lignin into High Octane Fuel Additives" by E. Chornet, D.K. Johnson, W. Zmierczak and J. Shabtai, has been accepted for presentation at the Conference on Progress in Thermochemical Biomass Conversion in the Tyrol, Austria, 17-22 September 2000.

The "Review of Market for Octane Enhancers" prepared by J.E. Sinor Consultants Inc., is in the review process so that it can be published as an NREL report.

Dr. Subha M. Kadangode has successfully defended her Ph.D. thesis "Lignin Conversion into Reformulated Hydrocarbon and Partially Oxygenated Gasoline Compositions" at the University of Utah.

Dr. Kadangode, as a student of Professor Shabtai's, performed a substantial amount of the work done at the University in support of the Lignin Conversion to Fuels task. Her thesis work was supported financially by the Biofuels Program through subcontracts with the University. Dr. Chornet attended her thesis defense and was part of her dissertation review committee.

## COLLABORATIVE INDUSTRIAL PROCESS DEVELOPMENT

### Summary of Technical Achievements or Results

We completed a series of dilute-acid pretreatment experiments for corn stover using the 4-L steam digester. Preliminary results indicate that because of the heterogeneity of corn fiber (i.e., a mixture of stalks, pith, leaves and cobs) the material was not pretreated evenly. Most noticeably, the stalks appear to be more resistant to pretreatment than the other components. Chemical analysis and enzymatic hydrolysis testing of pretreated samples to determine soluble-sugar yield and enzymatic-hydrolysis glucose yield are in progress. Preliminary results indicate the maximum soluble xylose recovery yield of 77% was obtained at pretreatment conditions of 180°C, 1% acid concentration in input material and 3-minute residence time.

A large batch of dilute acid hydrolysate was generated from Archer Daniels Midland Co.'s (ADM's) corn fiber using the 4-L steam digester. The soluble hemicellulosic sugar yield was 45 lb per 100 lb of corn fiber (or about 70% of theoretical yield). The hydrolysate produced from the steam digester has a total soluble sugar concentration of 197 g/L. However, approximately 40% of the soluble sugars are oligomers. The hydrolysate liquor was pressed from the residual solids, diluted to about 115 g sugar/L, then heated in a 2-gallon Parr reactor at 140°C for about 30 minutes to convert the oligomeric sugars to monomers. We sent a 1-gallon sample of hydrolysate liquor to ADM for further evaluation, and the remainder is being used for the strain development research at the National Renewable Energy Laboratory (NREL).

Our team completed a P-milestone entitled "Preliminary design of an ethanol plant based on two-stage dilute acid hydrolysis technology". The design is for a stand-alone 2,000 dry ton/day plant that converts California forest thinning to ethanol and electrical power. The estimated production cost of ethanol for the base case (i.e., based on laboratory data and no xylose fermentation) is \$1.22/gal assuming feedstock cost of \$25/dry ton and electricity selling price of \$0.42/kWh. In an improved case scenario where 75% process ethanol yield from xylose is assumed, the estimated ethanol production cost is \$1.03/gal.

We also completed a mechanical shakedown of the NREL-design 4-inch countercurrent screw extractor. This extractor will be used to generate data on hemicellulose extraction from pretreated biomass in a two-stage dilute acid hydrolysis process as well as single-stage dilute acid pretreatment process. This work is part of a larger effort in evaluating commercial solid/liquid separation equipment. The Aspen Plus model predicts that if the liquid-to-insoluble solid ratio (L/IS) in hemicellulose extraction of California forest thinning can be reduced from 4:1 (as used in the base-case) to 3:1, the production cost of ethanol would be lowered by about 5% (i.e., from \$1.22/gal to \$1.16/gal). Several exploratory extraction experiments were completed using pretreated California forest thinning. We achieved an L/IS ratio as low as 2.3:1. This is equivalent to a 1.8:1 liquid-to-total solid (L/S) ratio. Analysis is in progress to determine soluble sugar recovery. The target sugar recovery is better than 94% for an L/IS ratio of about 3:1.

## **General Technical/Scientific Progress**

### **Direct Industrial Support**

#### **ADM Corn Fiber Hydrolysate Production**

ADM requested NREL to generate a large batch of acid hydrolysate from corn fiber for strain developmental work. Even though protocols for producing corn fiber hydrolysates are available from the literature, NREL does not have equipment set up to generate the large amount of hydrolysates requested. Therefore, we carried out a series of hydrolysis experiments using the 4-L steam digester to establish the conditions that give about 45 lb of soluble sugars per 100 lb of dry corn fiber as obtained in the ADM reactor. Because the corn fiber supplied by ADM had been dried to 98% solids, considerable effort was required to develop a method for impregnating the dry corn fiber with acid without removing the water-soluble starch.

A large batch of corn fiber hydrolysate was produced using the steam digester. Approximately 40% of the soluble sugars are oligomers. The hydrolysate liquor was diluted and heated in a 2-gallon Parr reactor at 140°C for about 30 minutes to convert the oligomeric sugars to monomers. A total of 9 gallons of hydrolysate liquor was produced.

#### **Collins Pine/BCI PIER Project**

We reviewed a Co-products Validation Test Plan for the Collins Pine/BCI Project submitted by the Subcontractor (Enerkem Technologies, Sherbrooke, QC). Comments were provided to the Subcontractor. The final plan was approved by Collins Pine Co. Q. Nguyen, representing NREL, is the Technical Monitor for this subcontract.

### **Process Development**

The first of several series of preliminary dilute-acid pretreatment optimization experiments were completed for corn stover using the 4-L steam digester. The conditions include 160°C and 8-14 minute residence time, the normal operating range for the NREL PDU Sunds Hydrolyzer, so that the steam digester data can be used for scale-up experiments using the Sunds Hydrolyzer.

We are currently conducting experiments using the countercurrent screw extractor to generate data for developing an empirical equation for hemicellulose extraction from pretreated biomass. This work is part of a larger effort in evaluating commercial solid/liquid separation equipment. Similar models will be developed using data obtained from vendor tests. We will incorporate the extraction into the Aspen Plus Simulation Models for two-stage dilute acid hydrolysis process and enzymatic cellulose hydrolysis process as appropriate.

## **Scientific Publications, Presentations, and Other Activities**

### **Scientific Meetings**

Three poster abstracts were submitted to the 22<sup>nd</sup> Symposium on Biotechnology for Fuels and Chemicals. These are:

M.P. Tucker, Q.A. Nguyen, W.A. McHale, F.P. Eddy, K. Kadam, L.M. Gedvilas, and J.D. Webb.  
“FTIR Quantitative Analysis of Sugars and Lignin in Pretreated Softwood Solid Residue.”

K.H. Kim, M.P. Tucker, W.H. McHale, A. Aden, and Q.A. Nguyen “Continuous Countercurrent Extraction of Hemicellulose from Pretreated Wood Residues.” .

F.A. Keller, M.P. Tucker, F.P. Eddy and Q.A. Nguyen High-Yield Fermentation of Softwood Hydrolysate by Adapted Yeast followed by HPLC and FTIR Sample Analysis.”

#### **Patents or ROIs filed**

A patent application entitled “Strains of *Saccharomyces Cerevisiae* Yeast that uses Oxygen during fermentation of Normally Toxic Lignocellulose Prehydrolysate”, NREL IR #98-04 was submitted to the United States Patent and Trademark Office. The inventors are Fred Keller and Quang Nguyen.

## **PROCESS ENGINEERING AND DEVELOPMENT**

### **Summary of Technical Achievements or Results**

The Process Engineering and Analysis team completed several important studies in this time period. These studies are crucial to understanding the impacts of continued research on the cost of ethanol and other opportunities for biomass conversion. The team:

- Completed a technical review and summary of the results of four of the six Building a Bridge to the Corn Ethanol Industry subcontracts. Provided summary presentations to NREL and DOE staff.
- Completed a P Milestone report on the design of an ethanol plant based on 2-stage dilute acid hydrolysis technology.
- Completed a comprehensive market review of opportunities for fuels derived from lignin with J. Sinor Consulting.
- Expanded the work on the sugar platform in anticipation of supporting the Bioenergy Initiative.
- Completed several technical memorandum reports on; 1) cost of sugar for fermentation 2) wastewater treatment cost correlations 3) physical property update 4) Large Scale Facility Cost and Short Cut for Changing Facility Size 5) Denatured Fuel Cost Determination 6) Advanced Economic Calculation Spreadsheet 7) Conditioning and Solid/Liquid Separation (associated with conditioning) costs for Enzymatic Process

### **General Technical/Scientific Progress**

The team completed a technical review and summary of the results of four of the six Building a Bridge to the Corn Ethanol Industry subcontracts. We provided summary presentations to NREL and DOE staff. The final reports from the Merrick/High Plains, NYSTEC, Vogelbusch/Chief, and Purdue/Williams Energy subcontracts were presented and reviewed. Feed handling designs and costs were sent to Harris for review with the next step being inclusion into the NREL model for corn stover. The three contractors that evaluated co-location of a corn stover facility with a corn dry mill found that the enzymatic hydrolysis process was not cost effective. This finding was due in part to the fact that little of the dry mill plants' infrastructure could be shared when a genetically modified organism was used for the stover fermentation. Recommendations from the contract participants provided several avenues for further exploration of a co-location scenario; these are currently being evaluated.

We completed a P Milestone report on the design of an ethanol plant based on 2-stage dilute acid hydrolysis technology. A base case and several sensitivities were modeled using data from the softwood research team. The P-milestone expanded on prior milestone work (and a Merrick & Co. report) and focused on a 2000 BDT/day plant design. The laboratory research results highlighted a 59% glucose yield. The sensitivity analyses showed that plant size, feedstock cost, and xylose fermentation efficiency had large impacts on the 2-stage process. It also showed that the hemicellulosic sugar washing system could be operated more cost-efficiently at a lower liquid/solids wash ratio. Differences between Merrick's design parameters and NREL's model parameters were investigated as well. The main differences were shown to be hydrolyzer design/cost and IRR calculation basis (i.e. pre-tax vs. post-tax).

The team completed a comprehensive market review of opportunities for fuels derived from lignin with J. Sinor Consulting. The opportunity to convert the lignin from the biomass to ethanol process to an



octane enhancer, oxygenate, or high value fuel is based on the assumption that there will be a market for such products. Through a subcontract with Jerry Sinor, this assumption was investigated. We generated a thorough report on the current and future market environments and it will be issued as an NREL report. Regulatory issues, engine changes, and infrastructure were all addressed, providing a valuable tool to direct the lignin to fuels research.

The team expanded the work on the “sugar platform” in anticipation of supporting the Bioenergy Initiative. This work should help attract companies interested in other products and help kick-start the ethanol industry by proving and commercializing the production of sugars, from which ethanol production is less of a risk. We were able to refine the process of determining the original cost of fermentable sugars because we better understood how the process might provide low cost, dilute sugars for upgrading to a variety of products. The next step is to develop a biorefinery approach, evaluating a mix of products from biomass. The product mix would vary based on the markets, feed availability and composition, and overall plant economics. This concept allows for optimization of a slate of products, ranging from power to fuels to chemicals. Significant work needs to be done to understand the refinery concepts currently in practice in the petrochemical industry and apply them to the biomass feedstocks.

We made considerable progress with the U.S. Department of Agriculture (USDA)-Agricultural Research Service (ARS) office in Philadelphia toward a collaborative process design and costing report. The NREL rigorous Aspen model was simplified to be more user friendly in anticipation of an exchange of models with the USDA-ARS. The economics of the corn and corn stover process were compared and aligned, providing a check of each process’ design assumptions and costing data. We moved the power and equipment-sizing portion of the model to an Excel spreadsheet to make it more accessible to users. The comparison of the starch and cellulose processes will be summarized in a report in April 2000. We foresee doing further work to combine the processes into one plant to investigate the opportunities and hurdles of a co-location scenario.

During the period covered by this report, the subcontract with the Harris group was fervently underway. Several vendor tests on solid/liquid separation equipment were conducted with material generated from wood chips at NREL (post-fermentation) and TVA (prehydrolyzate slurry). The Lignin Upgrade project will use some of the extra material as feedstock for their research. The Harris group created a design specification for pretreatment reactors based on new corrosion data, generated as a part of the subcontract and input from NREL, and sent it to several equipment manufacturers. The resulting design and cost information from the vendors is to be used by Harris to determine the commercial viability of these reactors, as well as the type of testing needed for an engineering construction firm to provide a process guarantee. The counter current reactor, not specifically included in the original design spec, will be added to provide information for making the go/no go decision on continuation of its development. This decision is a sector milestone in the third quarter of 2000. Feedstock handling for corn stover is the other major area of research for the Harris subcontract. The contract completion date is uncertain at this point.

The bulk of the modeling for the 20-year outlook was completed and DOE performed a preliminary review. This project, envisioned to be an annual one, will help us understand the future of biomass conversion and keep the research focused on the long-term goal of a sustainable biomass industry. We modeled several outyear scenarios ranging from 2005 with enzyme improvements to multiple 2020 cases representing novel technology. A final version and report will be issued in June 2000.

Work on identifying the most appropriate corn stover composition for use in process design and economic evaluations began in earnest during this period. Previous data for hardwood used in the model was not well documented, so to avoid the same issues with stover, we initiated a concerted effort between the engineering team and analytical team. The plan is to collect stover data from a range of sources to try to identify the variations due to region, growing conditions, age, etc. Some representative value will be determined from this collection. This is expected to be an ongoing process, at least until there is confidence that we have adequately accounted for the major variations. We will also investigate the limitations of the analytical methods. Mass balance closure and better identification of the extractives in stover are important.

The team made initial contact with several pretreatment/hydrolysis research groups through the CAFI. The plan is to conduct economic evaluations of the different pretreatment technologies currently under study to provide more information on where the technologies are in development and what each can provide to biomass conversion.

The engineering team participated in a small business innovative research project with Reaction Engineering International to gather data on the drying and combustion of lignin residue from the biomass to ethanol process. The data collected was extremely useful and included vendor data and quotes for two types of dryers as well as emission data from a fluidized bed combustor. A phase II proposal has been submitted, which we are planning to support with engineering analysis. Collected data will be incorporated into the NREL model to improve its accuracy in the drying/combustion area, which uses estimated costs for drying and emissions.

The MYTP was refined and will be published in the third quarter of 2000. For FY2001 and subsequent years, the Annual Operating Plan (AOP) will be derived from the MYTP. This is a new goal, and is designed to improve the alignment of the AOP with the MYTP and ultimately with the DOE Biofuels program goals and plans.

We developed a preliminary gasification model to convert the lignin residue exiting the ethanol process to a synthesis gas. The residue is currently burned in a fluidized bed combustor to produce steam and electricity. This gas could be sent to a gas turbine for power generation or converted to a higher value co-product via fermentation or chemical conversion. This is a relatively new method of dealing with process residues and waste streams, initially used in refineries to convert their low value streams. Gasification is considered more environmentally attractive than combustion, but the costs are considered high and need to be offset by the product value.

The engineering team also provided data analysis and modeling support to the Arkenol and BRI CRADAs, and performed a pre-feasibility study for the Oregon Biomass to Ethanol project in support of the Partnership Development team.

## **Scientific Publications, Presentations and Other Activities**

Wooley, R. (1999). "Process and Economic Evaluation of Syngas Fermentation" Presented to Oklahoma State University's Biomass to Ethanol Research Consortium, October, 1999.

Nguyen, Q.; Aden, A. (1999). "Preliminary Design of an Ethanol Plant based on Two-stage Dilute Acid Hydrolysis Technology", P Milestone report, November, 1999.

Wooley, R. (1999). "Sugar Platform", Presentation to DOE Semi-Annual Management Review, December 9, 1999.

Wooley, R.; Ibsen, K.; Putsche, V. (1999). "Development of an Aspen Plus Physical Property Database for Biofuels Components", report addendum, December, 1999.

Wooley, R. (1999). "Modeling the Process Design and Economics of Biomass Conversion to Ethanol", Presentation to Department of Energy, Office of Fuels Development, December 14, 1999.

Aden, A.; Wooley, R.; Yancey, M. (January 2000). "Oregon Biomass to Ethanol Project: Pre-feasibility Study and Modeling Results", report to Bryan & Bryan, Inc. and the Oregon Office of Energy.

Wooley, R. (February 2000). "Rapid Evaluation of Research Proposals Using Aspen Plus", AspenWorld conference presentation and paper.

Ibsen, K.; Wooley, R.; Ruth, M.; Aden, A. (March 2000). "Using Databases to Streamline Process Design and Analysis", AIChE spring meeting presentation and paper.

## **LARGE SCALE FEEDSTOCK INFRASTRUCTURE**

### **Summary of Technical Achievements or Results**

The Large Scale Feedstock Infrastructure Task (LSF) is a new task initiated in FY 2000. The goal of the task is to support the commercialization of biomass-based fuels and chemicals by developing and disseminating supply, logistics, cost, and environmental information on biomass feedstocks. The Feedstock Infrastructure task will lead NREL Biofuels Program efforts to identify potentially viable feedstocks and collaborate with DOE, Oak Ridge National Laboratory (ORNL), Regional Biomass Energy Programs (RBEs), governmental agencies, industry, and other stakeholders to address feedstock issues necessary for use in a commercial facility. The LSF made two important accomplishments over the past six months.

- A C-milestone to revise the Corn Stover Plan was completed on schedule, January 2000. A revision of the current Corn Stover Plan was required to address industrial needs that have developed because of a more rapid deployment of technology using corn stover than what was originally anticipated by the planning team. A specific area that was identified in the Corn Stover Plan as critical is the identification and assessment of major issues surrounding the collection and handling of corn stover, and the development of strategies and infrastructure necessary to address those issues. As part of the Corn Stover Plan implementation, two approaches are being used to identify issues-- colloquies and life cycle analysis. Both of these approaches have been initiated along with other activities identified in the plan. The results of these efforts will be used to revise the Corn Stover Plan during the final half of FY 2000.
- The Corn Stover Colloquies were conducted during February and March 2000. A colloquy is a focus meeting design to provide two-way communications. Four colloquies between Biofuels Program representatives and corn stover stakeholders were held to address the current state of information surrounding corn stover collection and handling. A report on the results of the colloquies will be produced and reviewed by June 2000.

### **General Technical/Scientific Progress**

#### **Corn Stover Plan**

The original version of the Corn Stover Plan formalized the thoughts and strategies from an organizational meeting that was prompted by results of the Cellulase Colloquies. These results indicated that corn stover is a potential feedstock with a resource large enough to create interest among enzyme manufacturers. To incorporate advancements made in the plan and changes in the situation of corn stover as an ethanol feedstock, the plan was modified, and will continue to be a dynamic document until the major goal is accomplished.

To commercialize the use of corn stover to extend the biofuels' share of the transportation fuel market in a way that is economically viable and environmentally sound.

The plan targets the start-up of the first commercial ethanol plant that relies on large-scale collection and conversion of corn stover to ethanol. It does not project how rapidly the technology will penetrate the market place. This approach is consistent with prior program goals. It reflects the importance of this first-of-a-kind plant.

From the perspective of conversion technology development, corn stover use should not have huge impacts on plans outlined in the MYTP. For ORNL, looking at corn stover collection represents a much

more significant change compared to its classical research activities involving energy crops. Thus, while the corn stover plan covers all of the major activities that must be accomplished to realize a commercial scale plant using corn stover as its primary feedstock, it focuses more on new activities related to corn stover collection.

Marketing and deployment activities and conversion technology development are not presented in detail. Conversion technology development is covered more extensively in other planning documents. While marketing and deployment are critical to success in meeting the plan's goal, we see these as being the responsibility of as-yet-unknown future industrial partners. In addition, the plan does not address the development of high value co-products such as paper pulp from corn stover cellulose.

The effort to commercialize corn stover-to-ethanol technology is multidisciplinary in nature. ORNL and NREL are each primarily focussed on key aspects of feedstock collection and conversion, respectively.

But other organizations must also be involved. This plan identifies steps for outreach to the appropriate organizations and stakeholders. There are several key targeted stakeholders, such as the USDA and farm and commodity organizations such as the National Corn Growers. It is anticipated that the information generated by the efforts outlined in this plan will garner the support of these groups. Without their direct involvement reaching the plan's ultimate goal will be difficult.

The plan serves first and foremost as a shared vision for DOE, NREL, and ORNL. It is the first step in developing a shared vision that can be brought to others outside the Biofuels Program. Secondly, the plan serves as a means for coordinating the efforts at NREL and ORNL. Finally, this plan provides preliminary information on the timing, cost, and ownership for each of the major activities. It should provide DOE management with a clearer sense of the pathway that leads from our current situation to a future that includes commercial production of ethanol from corn stover.

Major efforts outlined in the plan include the following.

- We will work with ORNL and the Process Engineering Task to coordinate the development of a corn stover collection and handling model by ORNL that meets the technical requirements and information needs of the Process Engineering Task. This task will use the information to supply better feedstock cost data to their models and for a corn stover life cycle analysis.
- We will support the development of corn stover supply information. In FY 1999 a water erosion model was developed that provides information on the effects of agricultural residue removal on water erosion. In FY 2000 the task will support efforts to develop a wind erosion and soil tilth model to supplement the current water erosion model to identify where corn stover can be collected in an environmentally responsible manner. Maps showing suitability for corn stover collection will be produced using the soil erosion and tilth data.
- We will identify and develop relationships with major corn stover stakeholders. In addition, we will work with the USDA-ARS, USDA NRCS – NCGA/CRA, CTIC, the National Association of Conservation Districts, and the American Farm Bureau to address barriers to corn stover collection and communicate the benefits to potential producers.
- We will coordinate the Biofuels Program Feedstock Infrastructure research and communication efforts with DOE's Office of Fuel Development (OFD), ORNL, and RBEP feedstock research.

### **Corn Stover Colloquies**

Four colloquies were held in February and March 2000:

- Cedar Rapids, IA February 9, 2000
- Bloomington, IL February 16, 2000
- Lincoln, NE February 23, 2000
- Minneapolis, MN March 8, 2000

The colloquies were designed to obtain input from people positioned to be influential in a corn stover to ethanol industry. These individuals represented seven primary disciplines (Producers, Agriculture Equipment Suppliers, Life Science Companies, Ethanol Industry, Enzyme Industry, Agri-Pulp, and Engineering Firms). Care was taken to ensure that competitors were not invited to the same colloquy (i.e., John Deere and Case). In addition to the participants there were invited guests. Guests represented NREL, DOE, USDA, state agencies, environmental groups, and local economic development organizations.

The issues posed at each colloquy were producer benefits, collection and storage, economic process, and markets (ethanol and enzyme). These issues were addressed assuming that 30% of the corn produced can be collected in a sustainable fashion. Impacts of the proposed scenario were suggested to be:

- \$2.7 billion in farm income from feedstock sales
- A \$400-million enzyme market
- Sugars produced at less than \$4/cwt
- 6 to 8 billion gallons of ethanol
- Improved agricultural practices – less tillage and chemical use
- Mitigation of 12% - 20% US Kyoto commitment

A preliminary analysis of the results of the colloquies indicates that:

- Reliable supply of feedstock exist
- Lower cost collection, storage and handling methods can be developed
- Agri-pulp may provide synergies in a biorefinery concept with ethanol
- There are issues around the use of C5 sugars and fermentation by genetically engineered organisms

- Corn stover processing to sugar and ethanol will have a major impact on the rural economy, related industry, United States energy independence, greenhouse gas mitigation, and agricultural practices
- Education of targeted groups is needed
- Work on ethanol and enzyme market development is needed
- Additional research and development efforts are necessary to address issues around soil impacts, process economics, and development of higher value products
- The construction of a demonstration plant is needed to reduce risk and move the corn stover to ethanol to the next phase of development

A more detailed reviewed report is due in June as part of a C-milestone.

### **Life Cycle Analysis**

Efforts to assist the Process Engineering Team (PE) in the life cycle analysis for corn stover to ethanol were carried out during the first half of FY 2000. LSF organized several meetings between PE and Marie Walsh and Janet Cushman at ORNL. The purpose of these meetings was to coordinate the development of two major modeling efforts that will produce data for the life cycle analysis (macroeconomic model and logistics model).

Several meetings were held with representatives of the Natural Ecology Research Laboratory at Colorado State University (NERLCSU). In conjunction with USDA, NERLCSU has developed a model that estimates greenhouse gas balances in natural and managed ecosystems. NERLCSU can produce output that simulates greenhouse gas cycling under agricultural systems. These modeling capabilities and the NERLCSU contacts with USDA and the agriculture community can be very useful in the life cycle analysis effort. It is anticipated that a subcontract with NERLCSU will be placed early in the second half of FY 2000.

Four of the Bridge to Corn Ethanol subcontracts were completed in January and two more subcontracts were placed to complete the remaining studies. LSF provided oversight for these subcontracts for PE, and LSF has assisted in analyzing the results.

### **Soil Modeling**

The first of three phases to model important soil parameters in the major corn producing areas was completed in December 1999. The first phase consisted of a subcontract to assess soil erosion by water on a county level. The second phase was initiated in March 2000 and consists of a subcontract to assess soil erosion potential by wind on a county level. Both these assessments involve the use and modification of models developed by USDA to identify where corn stover can be collected using best practices to have no or minimal impact on soil erosion. The final phase will incorporate a tillage model to identify soil structure changes that may be caused by the removal of corn stover at specified levels.

### **Scientific Publications, Presentations, and Other Activities**

Wiseloge, A.E.; Hettenhaus, J.R. (2000). "Corn Stover to Ethanol" American Farm Bureau, Houston, TX, January 8-11, 2000.

# **BIODIESEL PROJECT**

## **Summary of Technical Achievements or Results**

The Waste Grease Composition Analysis with industrial partner Pacific Biodiesel (PB) has slowed due to cash flow problems at PB. Forty-five samples have been collected and processed by Univ. of Toronto (UT), a lower tier subcontractor. GC and ultimate analysis still need to be performed on these. If no significant variation is observed in these samples, an additional 45 samples may be cancelled.

The NREL Hydrogen program finished several experiments with biodiesel and wet glycerin (a byproduct of producing biodiesel). The hydrogen yield oscillated around 77% of the stoichiometric potential. It could be significantly higher if more steam were used in the reaction. Conversion of CO in the gas through water-gas shift to CO<sub>2</sub> and H<sub>2</sub> would increase the hydrogen yield to 95% of that theoretically possible. If feedstock is available for 5 cents/lb, the feedstock cost of hydrogen would be approximately \$3.30 GJ. A more complete analysis will be conducted later this spring.

University of California Davis completed bioassay analysis from the Multifeedstock Project. The results are under discussion at this time.

Environ has prepared biodiesel emission databases for use in EPA and CARB air shed models but the project is on hold until CARB issues its decision on what types of data they want to use in future modeling exercises. The discussion revolves around engine vs. chassis dyno data for heavy-duty vehicles. Chassis data may be too sparse to support modeling efforts.

## **General Technical/Scientific Progress**

### **Collaborative Activities**

A project initiated in FY1999 will explore the impact of using DTBP with biodiesel and B20 to eliminate or reduce NO<sub>x</sub> emissions. This work may be done in cooperation with USDA NCAR (Peoria) which has other additives identified for testing. In addition, USDA NCAR (Philadelphia) will be working with NREL to test novel formulations of "biodiesel". USDA will prepare several promising fuels and NREL will conduct property and emission tests. No disclosure of confidential information will occur at this time, although follow on work may require a memorandum of understanding or nondisclosure agreement.

CARB added biodiesel to their list of particulate control technologies. Any blend level of biodiesel can be used to reduce PM so long as the fuel meets all relevant CARB and CA regulations. NREL played a key role in those discussions and negotiations. One biodiesel producer has also acquired a fuel provider status under CARB for selling biodiesel in CA; that status confers CARB approval of biodiesel as a diesel substitute.

CSX railroad called NREL with an interest in loaning one of their locomotives for NREL's emission testing program at SWRI. Orange County Transit Authority may also be interested in loaning NREL a locomotive. Discussions are ongoing with both organizations at this time. The American Railroad Association is interested in the outcome of the project.

NREL is working with WestStart/CALStart and the U.S. Army TACOM division on a recycled grease biodiesel demonstration project. Approximately \$500,000 is available, and several white papers have been prepared and received favorably so far. A decision is expected this fiscal year.

NREL has offered to provide technical oversight on two projects that may provide opportunities to



improve biodiesel technology. One is a WRBEP proposal for biodiesel development in NV and CA, and the other involves biodiesel development in Puerto Rico (PR) with the DOE's Office of Diversity, OFD, and the PR Energy Office. The WRBEP project will conduct resource assessment in CA and NV and evaluate processes that reduce water consumption, expanding potential sites for biodiesel. The PR project will expand markets for biodiesel through demonstrations, evaluate process improvements for low cost recycled grease biodiesel, and document oil displacement benefits in PR.

A no-funds-in CRADA is under development with Biodiesel Development Corp (BDC) and NREL to evaluate the feasibility of a technology developed at UT and licensed to BCI. BCI is investing \$50,000 in development of laboratory data and NREL will fund the feasibility analysis and scale up design if sufficient data exists. UT has prepared a paper for presentation at Seville, Spain in June describing the process. The process appears to offer a route to continuous processing of recycled grease biodiesel but it is not clear that the process resolves the fatty acid conversion issue (yields typically only 96%).

The final phase of the Oxidative and Thermal Stability Test Methodology was on hold to find better fuel samples, since at least one initial sample was contaminated by the producer and anomalies were detected in another. The time and temperature testing will be repeated since the fuels received for testing failed to meet NREL's standards. The NBB has agreed to support the repeated testing. Fuel quality tests are underway on the new samples, and the project will be back on track shortly.

National Biodiesel Board (NBB) will submit the Tier II Health Effects report to EPA on May 21, 2000. NREL anticipates that the outcome will be favorable for biodiesel and NBB is planning a major PR campaign to promote the health benefits of biodiesel.

The Biodiesel Project is working with the Heavy Vehicle Technology Program to conduct EPA field task package heavy-duty emission tests of Fischer-Tropsch and biodiesel blends, and a PR has been prepared for release shortly.

The "Low Emission Diesel Engine Oil Workshop" held in Phoenix, AZ on January, 2000 was a success. Over 80 representatives of the oil industry, engine manufacturers and oil additive firms attended and the presentations were excellent. Joe Perez has submitted a final proceedings to NREL for publication. The proceedings will be published shortly.

The 3<sup>rd</sup> Annual Biodiesel Brainstorming Coordination Meeting has held in Phoenix, AZ in February, 2000. Over 40 representatives of universities, federal laboratories, DOE, USDA, TACOM, fuel injection manufacturers, and biodiesel producers attended. The presentations were excellent and generated very good discussions. R&D priorities were identified by the group and ranked in priority. Some longer term objectives were also identified. NBB coordinated the speakers and NREL provided the venue. S. Tyson presented the hybrid mustard project R&D targets.

CSM has completed their emission tests of biodiesel made from soy soapstocks as a joint partnership between NREL, USDA and CSM. Only 15 mil gal of biodiesel potential exists, so the effort is relatively small. The emissions appeared to be very similar to those of soy. USDA will work with the soy industry to commercialize this opportunity.

NREL and the Fats and Protein Research Foundation will jointly work on a Life Cycle Analysis of Yellow Grease Biodiesel Production. FPRF will provide industry data and NREL will support the modeling and report development in FY 2000 and 2001.

## **Scientific Publications, Presentations and Other Activities**

Other collaborative activities include:

- C S. Tyson attended National Biodiesel Board Meetings in February 2000. Feedstock neutrality was a major topic. The industry appears to be fighting over markets which are substantially smaller than their combined capacity. As a result, biodiesel prices (delivered to customers) have dropped to a range between \$1.25 and \$1.80 per gallon.
- C WRBEP meeting in Salt Lake City, UT, March 2000.

### **Other Presentations and Conferences**

December, 1999: DOE/OFD Semi Annual Update. Biodiesel R&D Targets.

### **Technology Transfer**

Event coordinators for Earth Day in Washington D.C. requested information on biodiesel. After consideration, approximately 85% of the power for the event (0.85 MW) was generated by reciprocating rig diesel generators powered on 100% biodiesel made from recycled grease provided by Griffin Industries and local petroleum distributors. The Kohler generators performed flawlessly. Kohler plans to promote biodiesel to other events this year (Kiss, PGA, etc.). The results of an engine tear down and inspection should be available in a few weeks.

Two thousand copies of a biodiesel Fact Sheet was prepared for Earth Day, which was a great success. A reprint of 2,000 was made. Fuel marketers have already requested almost 1,000 of those. The rest will be made available to trade shows and other venues.

S. Tyson made some comments about biodiesel on an National Public Radio news spot on April 21, 2000, just before Earth Day, which attracted a lot of interest at the Mall. Over 350,000 attendees were there at the peak of the event, and total estimates of attendees topped 500,000.

### **Project Operations**

Biodiesel R&D Targets: Feedstock (Phase 1). A hybrid mustard breeding contract with University of Idaho is in place and Anthony Turhollow of ORNL is providing technical assistance. NREL contacted several USDA offices, some midwestern farmers, EPA, NBB board members and several firms involved in biotechnology and pesticide development for informal feedback on the initial plans. Several very good suggestions were made to improve the existing contract. A contract modification will be complete this spring. A literature review is underway and a marketing study is planned for early FY 2001. In addition, an annual peer review panel is in the planning stages to provide technical support and guidance.

Biodiesel R&D Targets: Process Development. Three nondisclosure agreements for three biodiesel producers are being negotiated to allow access to production plants to improve the draft technology baselines for both soy and recycled grease biodiesel. The firms hope to be considered as industrial partners in future demonstration and process improvements in return. Several blue-sky evaluations of process improvements will be conducted in the summer of 2000. The review of other renewable diesel opportunities is nearing completion and should be available for 2001 AOP planning processes.

Four related projects, the IGT Multifeedstock project, the CSM NOx testing on biodiesel components, the Univ. of Iowa tests on speed of sound and bulk modulus, and the Univ. of CA Davis work on the bioassay results will be combined into a single report and published by the end of the year.

Colorado School of Mines continues work on the fundamental combustion of biodiesel with respect to NOx emissions.

The legal office has not resolved the removal of the biodiesel tanks from Chicago yet. The issue is still under investigation.

NREL has submitted a deposition in a challenge to the State of New Jersey public bid for biodiesel.

The bid requests only “virgin” “soy” biodiesel and expressly forbids the purchase of recycled grease biodiesel, and sets the cloud point for biodiesel in a manner that fails to protect the consumers of NJ and discriminates against some management options available to biodiesel and petroleum fuel providers to manage cold weather performance parameters. A similar challenge is under way in the State of OH, and with several utilities. Similar wording and typos have been noted in these discriminatory bids, leading some industry leaders to conclude that one firm is working with government purchasing officials and having undue influence in the design of the bids. One state official in NJ has been fired so far in the investigation of this matter.

A GSA biodiesel product definition has been issued, which is feedstock neutral and sets cloud point on the blended fuel used by the customer. Potential customers seeking appropriate bid language should be referred to the GSA product definition.

PR for “Petroleum Infrastructure Development” \$40,000 was competed. Educational activities with petroleum marketing firms.

Negotiated changes to “Oil Crop Potential for Biodiesel Production” with Univ. of Idaho XCO-9-29095-01. Impact on cost to be determined, likely is lower.

Modification of “The Impact of Biodiesel Fuels on Air Quality and Human Health” with Environ International Corp, AXE-9-29079-01. To fund remaining \$68,790 in FY 2000.